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Infection and Diet-Induced Gut Dysbiosis: Impact on Sleep Quality in Danio rerio

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INFECTION AND DIET-INDUCED GUT DYSBIOSIS: IMPACT ON SLEEP QUALITY IN *DANIO RERIO*

by

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ABSTRACT

A known bidirectional relationship between intestinal microflora and the central nervous system, coined the gut-brain-axis, has stimulated work on the association between gut dysbiosis and inflammation, and sleep quality. Previous studies in the Hayes Lab have reported that a high fat (HF) diet was correlated with immobile phases, a marker of low motility during sleep found in some neurological disease and sleep duration[1]. Long sleep duration (>1 S.D. above norms for age) is associated with poor sleep quality or sleep fragmentation in participants who are overweight or obese according to body mass index values[2]. The current work utilizes a reverse translational model to propose that we can identify some of the molecular mechanisms underlying consumption of a HF diet and sleep fragmentation. The animal model for the current study was *Danio rerio*, or zebrafish. Within this Honors Thesis, an existing protocol for *Danio rerio* sleep analysis was modified in order to assess the correlation between inflammatory pathways induced via direct infection by the human fungal pathogen *Candida* vs. HF diet-induced gut dysbiosis on developed measures of sleep quality[3]. Sleep analysis was administered via a Noldus DanioVision behavioral tracking device and showed that, in 80% of replicate experimental trials (n=86), infected zebrafish larvae exhibited increased total sleep duration, sleep % and mean sleep bout length. In 60% of replicate experimental trials (n=68), infected zebrafish larvae exhibited a greater number of sleep bouts, a finding consistent with sleep fragmentation defined as the number of sleep bouts divided by the total sleep duration. Sleep analyses conducted for diet-controlled larvae showed that zebrafish larvae administered a high fat diet exhibited an

increase in sleep bout number and larger fragmentation index, though this observation was not found to be trending or statistically significant. Zebrafish were euthanized and homogenized following sleep analysis in order to be used for single fish qPCR. In *Candida* infected larvae, gene expression of multifunctional pyrogenic and somnogenic cytokines TNF-α and IL-6 were upregulated according to -ΔΔCt values which are calculated as the negative difference of the ΔCt of each individual treated larvae and the average ΔCt value of each control, non-treated larvae. Similarly, in HF diet-treated larvae, gene expression of IL-1β and IL-6 was also elevated. Positive gene fold expression was elevated for IL-6 in both infected and HF diet treated zebrafish. These findings suggest that a mutual inflammatory pathway, triggered via direct infection or diet-induced gut dysbiosis, may exist for IL-6 mediated sleep disruption.

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INTRODUCTION

Sleep Physiology and the Molecular Clock

Sleep is defined as a behavioral state consisting of immobility and depressed responsiveness, both of which may be quickly reversed during the transition from sleep to wakefulness[4]. While it is unclear if all animals sleep, all higher eukaryotes not only exhibit regular sleep states, but also require sleep for proper maintenance of physiological processes[4]. Though sleep, a homeostatic phenomenon hypothesized to result due to the daily accumulation of adenosine primarily in the basal forebrain should not be confused with the distinct mechanism of circadian rhythm, a physiological 'molecular clock' which regulates higher eukaryotes to a 24 hour oscillatory cycle entrained to light-dark cycles via the suprachiasmatic nucleus or SCN[5, 6]. In fact, sleep-wake regulation is often described as a 'two-process model' defined by the homeostatic sleep promoting component (process S), which accumulates due to physiological energy expenditure and waste buildup throughout the day, and the circadian wake promoting component (process C) which serves to consolidate sleep periods and entrain sleep-wake states to light-dark cycles[7]. While a basic understanding of the neurophysiology underlying circadian rhythm and subsequent observable behaviors (*i.e.* nighttime sleep) is critical for understanding this project, this Honors Thesis is concerned with predicting homeostatic sleep features based on treatment by high fat diet and direct infection.

In humans, the foundation of the circadian rhythm (process C) begins in the SCN which has since been deemed the 'pacemaker' of the circadian clock[8]. In response to millions of years of evolutionary fine tuning, the SCN has become accustomed to daily environmental oscillations, such as sunlight and external temperature, such that it can regulate the body's general metabolic and physiologic profile to match the demands of the time of day. While the SCN and other neuroanatomical structures regulate the molecular clock at a body-wide level, each cell within the body is home to its own set of tightly regulated transcription factors which mediate the individual oscillations at a molecular and tissue trophic level. The largest transcriptional contributors to this phenomenon, and subsequently the most well understood, are Circadian Locomotor Output Cycles Kaput, Brain and Muscle Arnt-like 1, Period, Cryptochrome, Reverse Erythroblastosis Virus α, and Retinoid-related Orphan Receptor α or *CLOCK, BMAL1, PER, CRY, REV-ERBa,* and *RORa*, respectively[9]. While the roles and regulatory mechanisms of these genes and their encoded polypeptide products are quite intricate, their general function in maintaining circadian oscillations can be summarized quite simply. In short, proteins BMAL1 and CLOCK are known to dimerize and directly upregulate transcription of both *PER* and *CRY.* Following translation, PER and CRY are known to associate and re-enter the nucleus to then depress expression of their activators *BMAL1* and *CLOCK.* Moreover, *REV-ERBa* serves as a transcriptional repressor for *BMAL1* expression and *RORa* acts as a transcriptional activator[10,11]. In this manner, the core of the cellular molecular rhythm is regulated by BMAL1 and CLOCK which are externally regulated by REV-ERBα and RORα and internally regulated by their own downstream targets, CRY and PER. As *BMAL1* and *CLOCK* are intermittently activated

and repressed, the levels of PER and CRY oligopeptides gather within the cytoplasm and enter a complex signaling pathway resulting in melatonin production[12]. Thus, the core set of 6 proteins described above regulate the oscillation of melatonin, a critical somnogenic hormone, as well as a wide array of other hormone and protein communicators not described[8].

Circadian Regulation of Metabolism, Neural Phenomena, and Immune Activity

In addition to the melatonin-mediated sleep regulatory pathways discussed above, the cellular molecular clock system is also known to regulate general metabolic and physiological pathways involved in recovery, regeneration, and synthesis[13,14,15]. Perhaps the most well understood role of sleep in physiological development and restoration is the differentially observed release and fluctuation of circulating hormones. Specifically, growth hormone release, or the pulse of most anabolic hormones (*e.g.* testosterone and prolactin) for that matter, occurs during slow-wave sleep (SWS)[3]. In addition to this, catabolic hormone release (*e.g.* cortisol) is depressed during sleep states. Such restorative and growth oriented hormone release, coupled with the decreased energy demand during sleep, lends human sleep states (*i.e.* the result of process S described above in which the homeostatic driver of sleep induces immobility and depressed responsiveness) to play a necessary role in maintenance and regeneration.

In addition to endocrine associated bodily regeneration[14], the significance of metabolite clearance during sleep, specifically that of known neurotoxic waste product

amyloid, is well documented. For example, Xie *et al* reported that cerebrospinal fluid fluxes upregulated during sleep elevated clearance of β-amyloid, a waste product which accumulates as a result of typical neural function during the day[15]. Conversely, a key pathological finding of Alzheimer's Disease is excessive buildup of β-amyloid plaques and previous studies have shown that even Alzheimer's Disease patients have both elevated β-amyloid and circadian fragmentation as compared to cognitively normal individuals[16]. Both in age-related neurodegenerative diseases and in healthy individuals the importance of sleep in learning and memory has been extensively studied. For example, previous studies from the Walker group indicate that a full night's sleep yielded elevated motor learning and, in some instances, even properly timed naps were able to produce similar results[17]. Furthermore, related research has supported that an increase in nighttime sleep is correlated with learning efficiency, memory consolidation, and other neurological metrics associated with learned performance[18,19]. Complex experimental methods have reported direct correlations between sleep deprivation and impaired hippocampal-dependent learning suggesting that routine quality sleep is critical for neurogenesis, memory encoding, and spatial learning[20,21].

Most pertinent to this presented study is the interplay of the immune response and sleep loss. Specifically, even acute sleep loss (25-50% of a single 8 hour nightly sleep routine) has been shown to elevate basal inflammatory cytokine expression[22]. Moreover, many cytokines have been shown to hold somnogenic and pyrogenic properties meaning that they are involved in sleep as well as the pro-inflammatory response. The most well studied of these cytokines, tumor necrosis factor-α (TNF-α),

interleukin-1β (IL-1β), and interleukin-6 (IL-6), are all generally classified as proinflammatory proteins though differential expression of these cytokines is tightly associated with sleep. For example, all three proinflammatory signaling molecules are expressed at higher levels during sleep[23]. Similarly, anti-inflammatory cytokines which serve to downregulate the pathways activated by TNF-α, IL-1β, and IL-6 are upregulated during waking states and may even perturb the transition into sleep states[23,24]. Moreover, inhibition of IL-1β has been shown to induce sleep deprivation and decreased sleep rebound[23] and increased TNF-α expression has been linked to upregulated sleep duration[25], especially following infection[26]. To complement these associative findings, extensive work has been done in the field of chrono-immunology or the study of how circadian rhythms play a reciprocal role with immune activity. One such study found that in mutant mice strains with defective *CRY* genes, the repressor of *BMAL1* and activator of melatonin production described above, a more aggressive pro-inflammatory signaling response downstream of TNF-α activation was observed[27]. Finally, related publications report that even *Rev-erb*α partial knockdown (or deficiency) perturbs the oscillations of IL-6 production and knockdown or *CRY2* upregulates the production of IL-6[28, 29]. It is well understood that sleep, circadian rhythm, and immune activity are mutually regulated.

Sleep and Circadian Disruption as a Drive of Disease

Sleep is necessary for proper bodily function, particularly as it pertains to metabolic recovery, neurogenesis, and immune protection. perturbed sleep quality can

quickly lead to various pathological states. For example, patients with gastroesophageal reflux disease (GERD) who become sleep deprived due to any number of social or environmental factors have been reported to experience exacerbated pain and discomfort GERD symptoms[30]. Previous work indicates that obstructive sleep apnea (OSA) is known to induce, or at least worsen, hypertension[31]. Further, Porto and colleagues (2017) reported that extreme cases of OSA can even lead to myocardial infarction [32]. Interestingly, sleep deprivation can even lead to differential immune system function in pathological conditions. One example of this was reported by Palma and colleagues (2006) who suggested that sleep deprivation in mice models could induce early onset symptoms resembling the human autoimmune disease systemic lupus erythematosus[33]. Thus, disrupted sleep quality and circadian rhythm can, quite literally, cause the body to become accustomed to dysregulated immune function and self-harming pro-inflammatory signaling cascades. Preliminary results reported by Hayes *et al.,*2018, who examined the association between sleep fragmentation (defined as the sum of percent mobile and percent one minute immobile bouts divided by the number of immobile bouts during a sleep interval) and mild cognitive impairment in aging adults, are consistent with previous findings that age-related neurodegenerative diseases and both actigraphic and self-reported sleep loss are common[34, 35].

Disease as a Driver for Disruption of Sleep and Circadian Rhythm

The observation that sleep disruption may serve as a driver for human disease is not a unilateral phenomenon. Given that general body metabolism, neurorecovery, and

immune regulation are reciprocally regulated by oscillatory rhythms, many disease states are known to disrupt sleep and circadian rhythms[36, 37]. For example, previous studies have shown that various cardiovascular disorders may lead to disrupted circadian rhythm and associated symptoms[38]. Similarly, hypertension has been shown to dysregulate the circadian rhythm of natriuresis[39]. Most well studied, and most pertinent to this Honors Thesis, is the impact of age-related neurodegenerative disease on sleep and circadian rhythm. Namely, previous studies on the pathophysiological mechanisms of Alzheimer's Disease (AD) as well as the clinical prodrome of AD, Mild Cognitive Impairment (MCI), have determined that sleep and circadian rhythm disorders in MCI are common[40]. Related work has shown that aging persons with Alzheimer's display sleep disruption associated with elevated daytime sleepiness, elevated sleep duration, and elevated sleep fragmentation and individuals with MCI show profound sleep disturbances[41,42,43]. Given that MCI is defined as cognitive impairment that is not severe enough to impede daily functioning, often the earliest neuropsychiatric herald of MCI is poor nighttime sleep especially in both amnestic and non-amnestic MCI individuals[44, 45]. In particular, sleep fragmentation has been characterized as both a risk factor and a diagnostic metric for MCI which, as described below, would serve as the foundation current research conducted by Hayes[34,43].

Alzheimer's Disease, Mild Cognitive Impairment, and Sleep Disruption

Alzheimer's Disease (AD) composes 50-70% of dementia cases in North America[46]. It is projected that, by 2050, the global prevalence of AD will reach 106.4 million[47]. Mild cognitive impairment (MCI), amnestic type is a precursor disease to AD which presents as having cognitive decline which does not impede daily function [48]. Demographic and lifestyle factors including socioeconomic status, education, gender, and depressive symptoms have been correlated with MCI and ADRD disorders [34]. Amnestic MCI transitions to ADRD (Alzheimer's disease and related dementias) in 80% of cases within 6 years, developing novel methods of early detection at early stages is needed, as less than 40% of positive cases are identied in primary care or referred to a specialist. Sleep loss is an early sign of cognitive impairment and developing MCI in community dwelling aging adults[40]. Holtzman et al. proposes a bidirectional relationship between sleep continuity, particularly, stage 3 and 4 NREM, and amyloid and tau clearance that is widely supported by human and animal model findings as a potential mechanism for the role of sleep disorder in MCI/ADRD development[49]. Typically, diagnosing sleep fragmentation as a means of primary prevention for MCI requires the use of an overnight clinical sleep laboratory which is uncomfortable for aging patients, costly and often yields poor results. The Hayes Lab has developed a patented sleep recording mattress device that can be used in the home bed for detecting sleep-wake and respiratory patterns[34, 50].

The diagnostic utility of the sleep recording device is compared to FDA approved actigraphy watch (Philips Respironics, Actiwatch 2). Both devices identify bodily

movements and activity levels. Since then, computational analyses and algorithms have been optimized to characterize not only sleep and wakefulness but various sleep parameters including fragmentation indexes[51]. Within the context of the clinical trial, participants were acquired either by physician referral or community recruitment events. Each participant was then subjected to a 1 week at-home clinical sleep study consisting of sleep analysis, neurocognitive assessment, and a wide range of exploratory statistical analyses to elucidate other moderators of sleep quality such as dietary habits. On day 1 of the study, participants were administered a battery of neurocognitive assessments to be used for the physician diagnosis of neurotypical status, MCI, or AD and set up with both the home-mattress sleep recording device as well as the aforementioned Actiwatch positive control device. The sleep recording device and Actiwatch were picked up on days 3 and 8 of the study, respectively, and all neurocognitive and demographic metrics were compiled and assessed as potential moderators of any differential sleep variables observed between neurocognitive healthy aging participants and MCI or AD participants. It was hypothesized that greater actigraphic sleep fragmentation would be associated with MCI and AD in aging individuals. In addition to this, a pilot study was conducted which aimed to identify the power of dietary habits as a moderator of sleep quality. The primary function of the novel sleep recording device is to identify early MCI/ADRD in community dwelling adults using machine learning methods[52]. MCI-like sleep features showed chronic sleep fragmentation due to increased frequency and duration of *wake after sleep onset*, WASO), a finding supported by actigraphic findings in our sample and consistent with the MCI/ADRD literature. The two night at-home sleep analysis included a battery of neurocognitive assessments and sleep self-report instruments to identify

known moderators of sleep quality which have been previously symptomatic of age-related neurodegenerative diseases[49].

Western Diet and Sleep Quality in Aging Adults

In my Honors Thesis, I was particularly intrigued by the question of the impact of diet on cognition and AD. The specific mechanism is discussed in further detail below and in Figure 1 but, in brief, diet and aging have been associated with upregulated inflammation correlated with perturbed dendritic plasticity, altered neurotransmitter levels, and cognition[53]. As such, it is critical that more work be done to elucidate the molecular connection between nutritional intake and potentially-pathogenic inflammatory pathways. Specifically, little is currently known about the impact of Western Diet, characterized by high fat and sucrose intake (*e.g.* pre-packaged foods and high-fat dairy products) as well as low fiber consumption, on sleep quality in aging adults[54]. In this Honors Thesis, a self-reported dietary survey was administered to patient's already undergoing an at-home sleep study in order to attempt to identify an association between diet and MCI or AD-related sleep disruption. This pilot study was conducted on a subset of the participant population (n=13) who were administered The Dietary Fat and Free Sugar - Short Questionnaire (DFS)[55]. The DFS is a participant self-reported dietary questionnaire consisting of 26 scale ranked questions. Each question on the DFS pertains to the frequency of consuming certain foods, particularly those high in fat or free sugar. At the end of the metric, each question, scored 1-5, can be summated to yield a total DFS

score. Previous studies have optimized the DFS such that the total score can be used to place participants into one of two categories[55]. Participants who score less than 60 on the DFS are categorized as having nutritional intake within the World Health Organization's (WHO) recommended levels while participants who score greater than or equal to 60 on the DFS are categorized as having fat and sugar intake above the WHO's recommended levels. The DFS was administered on Day 1 of the one week at-home sleep study in order to place participants into 'Fat and sugar intake within recommended level' or 'Fat and sugar intake above recommended level' groups. It was hypothesized that participants who were placed into the 'Fat and sugar intake above recommended levels' group would display perturbed sleep quality, more consistent though not necessarily correlated with that of MCI and AD individuals than cognitively healthy individuals, as a result of gut-dysbiosis induced inflammation. Discussed further in the Results Section, the results of statistical analyses suggest that a diet consisting of high fat and sugar content is correlated with a greater mean number of immobile phases and longer sleep duration in aging adults suggesting that adults who consumed a Western Diet had more fragmented sleep and slept for longer.

Western Diet-Induced Gut Dysbiosis and Immune Disruption

While there are extensive social factors which may have mediated the association between poor diet and disrupted sleep quality (*i.e.* duration and immobility) in aging adults, there is also evidence to support that a more complex, inflammation-mediated

mechanism could connect the two behaviors. In particular, this correlation could be a result of gut dysbiosis and the gut-brain-axis.

It is estimated that, in every 1 gram sample of human gut tissue, there lives ~ 10^{11} bacterial cells[56]. It is well known that this bacterial population, commonly referred to as human gut microflora, participates in extensive intracellular signalling pathways which are necessary for human health. In particular, individuals with healthy immune systems display a diverse gut microflora population which is critical for nutrient metabolism, digestive health, and immune response communication[57]. Furthermore, gut microflora have also been shown to play a key role in systemic signalling cascades which may travel as far as the human central nervous system (CNS)[58]. Previous studies have shown that gut microflora are involved in CNS-related protective pathways which inhibit the symptoms of depression and anxiety[59]. Alternatively, various neurological disorders have also been shown to negatively impact gut microbiota-involved processes such as epithelial permeability and pathogen defense[60]. This bidirectional relationship between human gut microflora and the brain has been coined the gut-brain-axis (GBA).

Given that the human gut microflora and central nervous system are so tightly intertwined, it should come as no surprise that disrupting one's microbial gut health by consuming a poor diet can lead to damaging neurosignaling cascades. An example of this phenomenon in mice models can be summarized in the figure below[61].

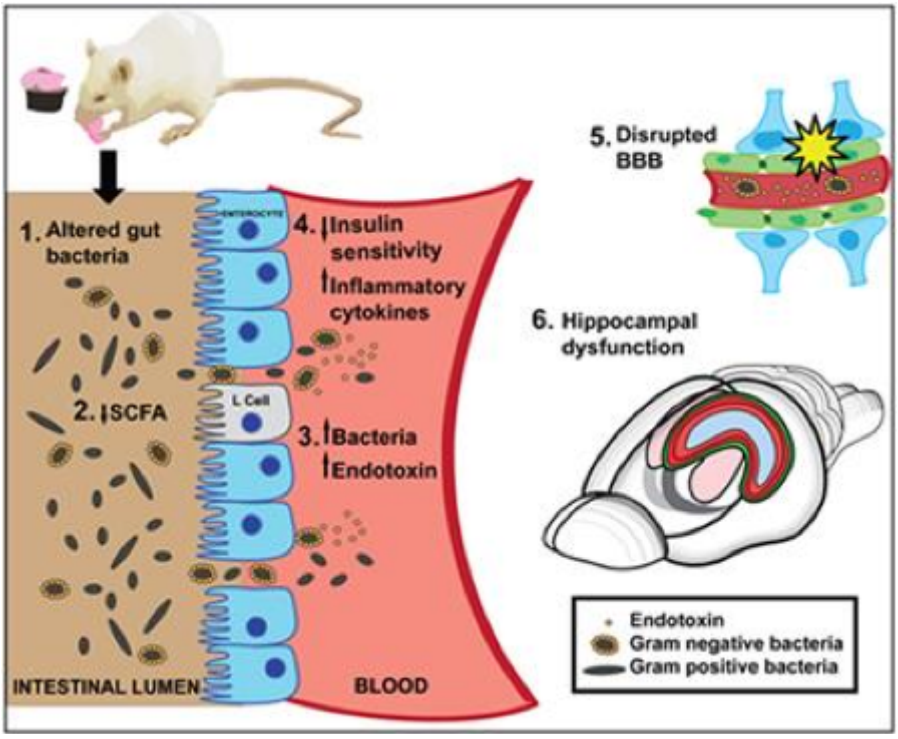

Figure 1. Schematic depicting how Western diet induces gut dysbiosis, systemic inflammation, and neurological pathology. (1) The mice is shown eating a cupcake, representative of a high fat and sugar diet, which alters the typical microflora of the gut as only select 'good bacteria' can survive on such poor nutritional intake and some 'bad bacteria' or opportunistic pathogens invade the inhabitable epithelia. **(2)** As this occurs, there is an overall change in the biochemical profile of the gut including alterations such as depressed levels of short chain fatty acids among others. **(3)** As commensal bacteria cells die, they release endotoxins stored within their lipopolysaccharide layer which are absorbed through the intestinal epithelium, triggering an immune response. **(4)** As a typical component of the acute phase response, pro-inflammatory cytokines are released to recruit immune cells to the gut. **(5)** These pro-inflammatory cytokines are not gut-tissue trophic and can travel via the blood brain barrier to the central nervous system. **(6)** Hippocampal disruption occurs, along with other general CNS disruption and

inflammatory signal cascade induction, which leads to a diseased state. Figure published by Noble *et al.,* 2017).

As described above, a key characteristic of high fat diet fed mice is depressed levels of short chain fatty acids (SCFAs) which are produced as a byproduct of bacterial metabolism as many commensal microbes ferment carbohydrates that cannot be digested by host cells. These microbial produced SCFAs (*e.g.* butyrate) mediate development of regulatory T cells in the gut which are necessary for mitigating intestinal inflammation. Furthermore, these same SCFAs have been shown to bind to specific G protein coupled receptors on the surface of neutrophils to perturb their crossing into the intestinal epithelium which results in inflammation. As such, previous studies have shown that just high fat diet consumption can alter gut microbe inhabitants in a manner that limits the amount of inflammation-preventing fatty acids, thereby leading to gut-dysbiosis induced inflammation[62]. Further, related research has found that such SCFA-mediated T cell development may serve as the tipping point for polarization of naive T cells towards T regulatory cells or Th1 and Th17 cells. In addition to this, SCFAs have also been shown to inhibit pro-inflammatory markers (*e.g.* TNF-α and IL-6) in macrophages[63]. As a result, depressed levels of intestinal SCFAs tend to elevate pro-inflammatory cytokine expression, elevate pathogenic T helper cell presence, and suppress T regulatory cell presence which was found to induce central nervous system inflammatory cell infiltration, elevate demyelination, and promote axonal damage[64].

In concordance with the hypothesized mechanism described above which explains how a high fat diet can lead to disrupted SCFA presence, altered immune cell

development, and disrupted CNS maintenance, related work by Lee *et al.,* (2017) further characterized the impact of an obesogenic diet on differential gut microbial inhabitants and subsequent impaired gut immune health. In this study, Lee and colleagues fed mice model groups with a normal chow diet or a high fat diet before various histological, cell culture, and molecular methods were used to identify the putative harmful effects of a Western diet. Beginning with the histological changes, Lee and colleagues observed that high fat diet treated mice contained morphologically altered Paneth cells, which line the intestinal epithelium in the small intestine and regulate commensal microbe growth via secretion of antimicrobial peptides. Moreover, the remaining Paneth cells in high fat diet treated mice contained less eosinophilic granules and produced less lysozyme and procryptidin, among other antimicrobial peptides (*e.g. Defcr1*). Furthermore, Lee and colleagues also identified via confocal microscopy and RT-qPCR methods that high fat diet treated mice had less goblet cells within the interstitial epithelia and subsequently produced less mucin, a key protein involved in the maintenance of the epithelial mucus membrane which binds to various gut microbes in order to prevent their crossing of the epithelial barrier. Such epithelial crossing by immune cells, as well as bacterial lipopolysaccharide (*i.e.*bacterial translocation) has been further shown to upregulate pro-inflammatory markers such as (TNF-α, IL-1, and IL-6) in the gut and other tissues (*e.g.* liver and pancreas)[65]. Finally, 16s rRNA analysis identified that high fat diet treated mice had a greater abundance of *Firmicutes* and *Actinobacteria* as well as depressed levels of *Bacteroidetes*. In this manner, Lee and colleagues observed that a high fat diet altered the intestinal epithelial cell profile, particularly in cells involved in commensal microbe tolerance and gut immune activation, impaired the function of protective mucus

layer-producing goblet cells, perturbed the barrier function of the gut, and directly altered the presence and abundance of select gut microbes. As such, previous work suggested that mice treated with a high fat diet experience gut-dysbiosis associated with altered gut microbiota and intestinal immune health which has been known to induce altered immune cell differentiation, epithelial barrier function, and even central nervous system activity[66].

While the phenomenon described above of Western diet induced gut dysbiosis links together an obesogenic diet, altered gut microbiota presence, immune activity, and CNS function, no studies to date have defined how this mechanism may directly impair sleep quality. The clinical observation which supported that individuals who consumed diets high in fat and free sugar showed a greater mean number of immobile phases and sleep duration, could be an empirical example of the phenomenon described in Figure 1 and highlighted in the studies discussed above. Thus, the human sleep data generated for MCI and cognitively normal participants raised a question of the physiological mechanism connecting poor diet to disturbed sleep quality in aging adults. In order to examine this question in a tractable vertebrate experimental system, zebrafish were selected as a model organism.

Zebrafish as a Model Organism

Zebrafish are cyprinoid teleosts which are quickly becoming a popular model organism for biomedical research[67]. In addition to being cheap to purchase, zebrafish husbandry and aquarium spawning is relatively simplistic. Though they can begin eating as early as 3 dpf, zebrafish larvae do not *need* to be fed for long-term survival until 4 dpf, allowing researchers to save time and money on general maintenance and husbandry[68]. In addition to this, adult zebrafish can spawn in under 24 hours allowing for researchers to rapidly collect fresh larvae even multiple times a day. In regards to molecular experimental methods, as it pertains to this study, the zebrafish and human genomes share 71.4% homology, particularly in regards to the innate and adaptive immune system[69]. As such, zebrafish can be used as safe alternatives for genome editing analyses in order to model human disease and pathogenesis. In addition to this, mutant zebrafish strains with pre-determined genetic mutations (*e.g.* TNF-α knockdown) are commercially available. Zebrafish larvae are also naturally transparent until about 5 dpf which is useful for various microscopy, injection, and fluorescence-based assays[67]. Thus, this model organism is perfectly suited for modeling reverse translational research of human disease as described in this study.

Zebrafish as a Model Organism for Sleep Analysis

In mammalian species, two distinct categories of sleep have been identified: rapid eye movement (REM) sleep and non-rapid eye movement (NREM) sleep.

Interestingly, zebrafish and humans both share nearly identical sleep ontogeny profiles conserved through evolution[3]. Similarly, in both humans and zebrafish, as age increases sleep percentage decreases. With regard to other common sleep variables, including sleep percentage, sleep bout length, sleep bout number, and fragmentation, zebrafish and humans show similar results. However, while sleep-wake transition counts do not necessarily decrease as humans age, they do decrease in aging zebrafish[3]. Thus, zebrafish larvae are likely the best model for reverse translational research examining any sleep human sleep variable as they are the most comparable in regards to overall sleep ontogeny. Finally, much like humans, zebrafish sleep quality has been shown to be impacted by environmental factors including temperature and light changes[70]. Thus, zebrafish circadian rhythm can be entrained under desired lab control conditions in ways that would not be possible with human participants, allowing for analysis of moderators such as diet and direct infection.

Zebrafish as a Model Organism for Immune Response Analysis

As discussed above, zebrafish have become a popular model for general biomedical research as their genomes can easily be mutated to model human disease, they mate rapidly to produce large spawn batches, and have high genomic homology to humans[71]. Moreover, by 4 days post fertilization (dpf), zebrafish larvae exhibit fully robust T and B cell development which contributes to adaptive immunity[72]. Prior to this, the innate immune response is fully developed and able to be manipulated for

experimentation. In addition to such professional immune cells, immune signaling cytokine messengers serve homologous functions in both humans and zebrafish. Specifically, the pyrogenic and somnogenic cytokines most pertinent to this study, TNF-α, IL-1β, and IL-6, are mutual between humans and zebrafish[73]. Zebrafish larvae have been used extensively as a model organism for fungal infection analysis, including that of *Candida* with previous research indicating that *Candida* infection upregulates TNF-α and IL-1 production as observed in humans[74,75]. Thus, zebrafish are a suitable candidate to model the positive control in the present study for a pro-inflammatory state induced via *Candida* infection.

Zebrafish as a Model Organism for Dietary Habits and Diet-Induced Gut Dysbiosis

Zebrafish larvae can be fed as early as 3 dpf with required feeding beginning at 5 dpf[68]. The impact of various dietary profiles on zebrafish health has been extensively studied to model human nutrition and nutritional genomics[76]. In addition to this, following dechorionation at 1-2 dpf, zebrafish are exposed to the non-sterile environment which mediates the development of normal gut microflora colonization as the intestinal tract fully matures between 3-4 dpf. Thus, zebrafish have been used extensively to assess the impact of diet on gut-dysbiosis. Previous studies by Arias-Jayo and colleagues (2018) reported that a high fat diet induces gut microbiota dysbiosis as well as intestinal pro-inflammatory signaling in zebrafish larvae[77]. Moreover, previous studies have also reported that even short-term high-fat diet exposure can induce cognitive decline in

zebrafish, suggesting that such gut dysbiosis reported by Arias-Jayo and colleagues could lead to pro-inflammatory signaling-mediated central nervous system disruption[78]. Thus, zebrafish are a qualified model to assess the impact of a high fat diet on diet-induced gut dysbiosis and subsequent sleep disruption in the current study. In this Honors Thesis, it was hypothesized that zebrafish treated with a high fat diet would exhibit gut dysbiosis-induced inflammatory gene expression resulting in sleep disruption. The aims of this study are as follows:

1. To determine if a high fat diet induces differential sleep quality, particularly as it pertains to fragmentation and sleep duration, in the common model organism *Danio rerio*, or zebrafish.
2. To determine if direct infection by human fungal pathogen *Candida albicans*, proposed as an alternative method of mimicking the putative inflammatory state triggered by high fat consumption, induces sleep disruption in zebrafish.
3. To determine if any differential sleep quality observed in zebrafish experiencing inflammation, be it by consumption of a high fat diet or direct infection, is correlated with elevated pyrogenic and somnogenic cytokine expression (*i.e.* TNF-α, IL-1β, and IL-6). It is anticipated that *Candida* infection will induce greater sleep disruption than high fat diet administration due to stronger pro-inflammatory cytokine signaling.

Overview

This Honors Thesis reports a reverse-translational study which assessed the impact of high fat diet and infection on sleep quality in zebrafish. The preliminary data reported within this Honors Thesis pertains to results I generated in a pilot study conducted within the MCI/ADRD ongoing study. In short, with Dr. Hayes' assistance in amending the parent IRB, I was able to examine the correlation between Western Diet in MCI diagnosed participants and actigraphic sleep. In those data, sleep duration and immobility during sleep was positively correlated with DFS measure of high fat/high carbohydrate foods typical of the Western Diet. I hypothesized that in aging adults these sleep features were mechanistically linked to gut-dysbiosis induced inflammatory signaling. In order to address this hypothesis, a follow-up study was designed and conducted in zebrafish in order to identify if pro-inflammatory cytokine expression, induced via diet or infection, was a moderator of sleep duration and immobility. A trend for a correlation between *Candida* hindbrain infection, sleep disruption, and elevated TNF-α and IL-6 gene expression was observed . Further, a trend for a correlation between high fat diet, sleep disruption, and elevated IL-6 gene expression was observed.

MATERIALS AND METHODS

I. At-Home Sleep Study Experimental Model and Human Participant Details

Participant recruitment

Participants of this study were acquired via community volunteers and physician-diagnosed MCI patients. Each participant underwent a two-day sleep study followed by a one month follow-up neurocognitive assessment. For each sleep study, data was collected within each participant's home utilizing a non-invasive sleep mattress device. This device is capable of collecting both respiration and movement data necessary to determine sleep fragmentation. Each participant was asked to maintain their typical sleep routine for the duration of the study. Participants within this pilot study (n=13) represent a group of individuals taken from the larger study described above (N=95). All participant interactions were approved by the UMaine Institutional Review Board for the Protection of Human Subjects (IORG #: IORG0000642). See Appendix A for IRB Approval Letter.

II. The Dietary Fat and Free Sugar - Short Questionnaire

On Day 1 of the two-day sleep study, the Dietary Fat and Free Sugar - Short Questionnaire, or DFS, was administered to each participant and scored according to the

protocol developed by Francis and Stevenson, 201[55]. The DFS is a self-reported dietary questionnaire with 26 questions. The first 24 questions assess weekly intake of specific foods (*e.g.* pizza or chocolate.) The last two questions assess yearly take-out food intake and weekly free sugar intake. A copy of the DFS can be found in Appendix B. Each question is answered numerically 1-5 with a score of 1 indicating once per week or less and a score of 5 indicating five times per week or more. The total DFS score describes the sum of all answers 1-26 with a minimum total score of 26 and a maximum total score of 130. Francis and Stevenson, 2019 defined a total DFS score of 60 or greater as indicative of a total nutrient intake above recommended levels reported by the World Health Organization[55]. In addition to the DFS, demographic information and past medical history were obtained for each participant as a routine component of the core study.

III. Actiwatch and Activas Diagnostics Sensor Actigraphy Collection

All participants were subjected to sleep analysis by the UMaine Psychology and Engineering lab teams (PI: M. Hayes, PhD; A. Abedi, PhD) as well as a positive control actigraphy watch (Philips Respironics, Actiwatch 2) which had been previously approved by the American Academy of Sleep Medicine[74]. The non-invasive home sleep monitoring system developed by the UMaine teams utilized 32 sensors to record sleep movements and respiratory rate. In order to distinguish between brief whole-body movements (sleep movements) and gentle, sleeping respirations (respiratory rate), the

sleep recording system consisted of both high weight (50 lb) and low weight (1 lb) sensors which were stacked on top of one another and adhered onto a typical cotton bed sheet in sixteen locations, totalling at 32 embedded sensors. In addition to recording two nights of sleep monitoring data using the UMaine sleep device, the actigraphy watch was worn by each participant for 7 consecutive nights. Each participant was instructed to wear the watch on their non-dominant hand at all times for the one week period aside from showering and certain activities which may have damaged the device. As opposed to the multi-sensor system utilized by the UMaine sleep device, the actigraphy watch coded sleep and wake states in 1 minute epochs through an accelerometer sensor located on the bottom of the watch. At the end of the one week period, the control actigraphy watch was collected by the UMaine teams and sleep/wake data was analyzed by an optimized program produced by the watch manufacturer (Philips Respironics, Philips Actiware 6: v6 0.9).

Table 1. Human participant actigraphic variable and definitions.

Actigraphic Variable	**Description**
Time in Bed	Time elapsed between the start and end of the rest interval
Assumed Sleep	Time elapsed between the start and end of the sleep interval
Actual Sleep Time	The total number of epochs for the given interval scored as sleep by Actiware multiplied by the epoch length in minutes
Sleep Percent[a]	The percentage of epochs in an interval that are scored as sleep. Scored total sleep time divided by (interval duration minus total invalid time (sleep/wake)) multiplied by 100
WASO/Wake Time	The total number of epochs between the start time and the end time of a given interval (sleep) scored as wake by Actiware software multiplied by the epoch length in minutes

Table 1 continued

Wake Percent[a]	The percentage of epochs in an interval that are scored as wake. Scored total wake time divided by (interval duration minus total invalid time (sleep/wake)) multiplied by 100
Sleep Efficiency	The percentage of time spent in bed sleep scored total sleep time divided by (interval duration minus total invalid time (sleep/wake)) multiplied by 100
Sleep Latency	The time required for sleep to start after initiating the intent to sleep. The time between the start of a given rest interval and the sleep interval start time, in minutes, and is controlled by the sleep interval detection algorithm
Sleep Bouts[a]	The total number of continuous blocks of epochs where each epoch is scored asl sleep for the given time interval (sleep)
Wake Bouts[a]	The total number of continuous blocks of epochs where each consecutive epoch is scored as wake for the given interval (sleep)
Sleep Bout Time[a]	The scored total sleep time divided by the number of sleep bouts for the given interval (sleep)
Wake Bout Time[a]	The scored total wake time divided by the number of wake bouts for the given interval (sleep)
# of Minutes Immobile	The total number of epochs in the given interval scored as immobile by the actiware software multiplied by the epoch length in minutes
Immobile % Time	The percentage of epochs in the given interval scored as immobile. Scored total immobile time divided by (interval duration minutes total invalid time (activity)) multiplied by 100
# of Minutes Moving	The total number of epochs for the given interval scored as mobile by the actiware software multiplied by the epoch length in minutes
Moving % Time	The percentage of epochs in the given interval scored as mobile. Mobile time divide by (interval duration minus total invalid time (activity)) multiplied by 100
# of Immobile Phases	The total number of continuous blocks of epochs where each epoch is scored as immobile for the given interval
Mean Length Immobility	The scored total immobile time divided by the number of immobile bouts for the given interval (sleep)
1 Minute Immobility	The number of immobile bouts that are one minute in length for the given interval (sleep)
1 Minute Immobility %	The percentage of immobile bouts that are one minute in length for the given interval (sleep)
Total Activity Score	The sum of all valid physical activity counts for all epochs for the given interval (sleep)

Table 1 continued

Mean Activity Score	The average of all valid physical activity counts for all epochs for the given interval (sleep)
Fragmentation	An index value that includes mobility and short sleep bouts. The sum of percent mobile and percent one minute immobile bouts divided by the number of immobile bouts for the given interval (sleep)
Mean Activity/min	The average of all valid physical activity counts for all epochs for the given interval (sleep) divided by the epoch length in minutes
Std AC	The standard deviation of all valid physical activity counts for all epochs for the given interval
Max AC	The largest valid physical activity value recorded during the given interval
Snooze (Sleep Time)	The time required to become active after sleep end. The time between the end of a given sleep interval and the end of the rest interval, and is controlled by the sleep interval detection algorithm

Note.
[a]Indicates Sleep Variable holds translational power to zebrafish larvae.
[b]Indicates Sleep Variable holds translational power to zebrafish but is calculated differently between species.
Reference. Philips Respironics Actiware (v6 0.9), Actiwatch 2tm, Respironics 2018.

IV. Human Participant Statistical Analysis

All participant information was de-identified and entered into SPSS software (SPSS Inc., 2007) for statistical interpretation. Participants were separated into two groups denoted “Fat/sugar intake within recommended levels” and “High fat/sugar intake”, according to the proposed cut-off score of a total DFS score above 60 likely indicating nutrient intake above the World Health Organization’s recommended levels[55]. Table 4 shows the group determination of the participants in this study. Sleep coding and actigraphy interpretation was conducted by the engineering and graduate team

of the Hayes Lab. Independent sample T-tests were run in order to analyze the association between fat/sugar intake and actigraphy output.

V. Experimental Model and Animal Subject Details

For all infection and sleep analysis experiments, AB male and female zebrafish larvae (University of Maine, Zebrafish Facility, Orono, ME.) were used with IACUC approval and regulations (Protocol Number: 12018-10-01, # Approved: 4825).

VI. Zebrafish Husbandry

All zebrafish larvae were placed in solution of E3 supplemented with 0.03% of 1% methylene blue (v/v) from time of collection to 24 hpf (*e.g.* 50 mL E3 with 16.5 µL of methylene blue or 150 mL E3 with 50 µL). Total larvae counts of ≤ 50 or less were kept in 50 mL E3 and total larvae counts of ≤ 150 were kept in 150 mL E3. It should be noted that larvae were not treated with *N*-Phenylthiourea to prevent melanization as the natural pigmentation of the larvae was required for the DanioVision device to properly track the larvae during the sleep analysis protocols described below. At 24 hpf, larvae were transferred to 100% E3 solution of the same volume described above and dechorionated with fine tweezers. All petri dishes containing larvae were stored at 28 ºC, pH 7.2, with 14 hour light/10 hour dark cycles regulated by a C by GE On/Off Smart

Plug (GE Lighting Inc.). E3 solution was replaced daily and larvae viability was assessed via discoloration (*i.e.* white, cloudy appearing larvae were presumed dead and removed from solution to be euthanized).

VII. Zebrafish Dietary Preparation

Zebrafish dietary preparation was performed as modified from Arias-Jayo and colleagues[72]. For control feed, 1 g of Microgemma 75 (Skretting Zebrafish, 2021.) was dissolved in 100 mL deionized water via heated stirring. For high fat feed, 0.9 g of Microgemma 75 and 0.1 g of pure Cocoa Butter (Mary Tylor Naturals) was dissolved in 100 mL deionized water via heated stirring. Both control and high fat feed were autoclaved and allowed to cool before feeding periods. Feeding periods occurred once per day at 6 pm, or the halfway point of the 10 hour light cycle. Larvae were fed 12.5 mL of feed in 150 mL petri dishes for 10 min periods. Control and high fat designated larvae were moved to clean petri dishes with fresh E3 following feeding periods. Between feeding periods, the control and high fat feeds were stored at 4 ºC in a dim light.

VIII. Zebrafish Hindbrain Ventricle Infection

Candida Culture Preparation

The following steps were conducted in collaboration with Bailey Blair. Zebrafish hindbrain infection was performed as modified from Brothers and colleagues (Brothers *et*

al, *Immunology and Infection*, 2012). When the to-be-infected larvae were at 1 dpf, an overnight culture of *Candida albicans* SN250 (Homann et al, 2009) in 5 mL 2% YBD media was prepared from a pre-prepared streak on LB agar plates.

Injection Preparation and Hindbrain Infection

The following steps were conducted in collaboration with Bailey Blair. The morning prior to larvae hindbrain injection (2 dpf), 500 µL of overnight culture was placed into a 1.5 mL microcentrifuge tube and centrifuged at max speed for 30 sec. The supernatant was discarded and the pellet was resuspended in 500 µL of PBS. The remaining solution was then treated with 37.5 µL of Calcofluor white stain (Sigma-Aldrich, #18909) and incubated at RT for 5 min. The now stained cells were then washed three times by addition of 450 µL PBS and centrifugation at max speed for 1 min with the supernatant discarded between steps. The stained and washed culture was then serially diluted by pipetting 50 µL into 450 µL of PBS to a final dilution of 1×10^{-2} cells/mL. Next, 10 µL of the final dilution was counted via hemocytometer and diluted with PBS to a final volume of 1×10^{7} cells/mL. A micropapillary injection needle was equipped with a micropipette puller device. The needle was broken using fine tweezers to create a shorter needle tip more optimized for hindbrain infection and to optimize the drop size of the needle (*i.e.* a drop of 1 hemocytometer 1 mm x 1 mm square was preferred). The micropapillary injection needle described above was loaded with *Candida* injection culture, or PBS for control larvae, before being mounted onto a micromanipulator. At 24 hpf, the embryos were placed in an anesthetization buffer of non-lethal Tricaine (4

mg/mL) ~10 min before hindbrain injections were completed. Anesthetized embryos were then moved to a Petri dish with a thin layer of 1% agarose to prevent embryos from sliding around during infection. A hair loop tool was used to orient the embryos in an appropriate location for hindbrain injection (*i.e.* dorsal side facing towards the needle tip). The needle tip was inserted into the hindbrain ventricle from this position and injection was confirmed to be in the proper location if a rise in the general anterior hindbrain area was observed.

Infected Zebrafish Screening

The following steps were conducted in collaboration with Bailey Blair. *Candida* infected embryos were placed in 96-well plate (wells B2-G11) in E3 and analyzed via Vivotome microscope at 10X magnification, with TL and RL Illumination On, Reflector 34 BFP, and standard Light path settings. After focusing on each fish, the fine zoom knob was used to scan through different cuts of each embryo. Embryos with 10-25 fungal cells (*i.e.* illuminated blue spheres) were kept for sleep analysis and qPCR, embryos above or below this range were euthanized.

IX. Zebrafish Sleep Analysis

DanioVision sleep analyses were performed as modified from Sorribes and colleagues[3]. At 5dpf, during the last 2 h of the respective dark cycle, each zebrafish

larvae was placed into a single well of a 12-well plate in 3 mL of E3 solution. The covered 12-well plate was then loaded into the DanioVision device to perform sleep analyses (Noldus Inc., . Larvae within the 12-well plate were transported within a styrofoam box to minimize light exposure during the tail end of the dark cycle. Each larva was then subjected to a 30 min sleep analysis within the DanioVision with a 5 min acclimation period. The DanioVision device was pre-programmed to the following conditions: Number of tracking sources: 1; Center-point detection (Activity analysis); Arenas: 12. Unit of distance: cm; Unit of time: s: Unit of rotation: deg. All data collected was directly downloaded within the EthoVision XT program (Noldus, EthoVision XT,) and exported to Excel for further analysis as described below. Single larvae were either euthanized following sleep analyses or prepared for single fish qPCR as described below.

X. Zebrafish qPCR

RNA Purification

Directly following sleep analysis, the 3 mL of E3 within each larvae-containing well of the 12-well plate was replaced with a euthanization solution (*i,e.*10 mL E3, 20 µL Tris buffer (pH 9.0), 10 µL lethal Tricaine (400 g/L)) and allowed to sit at RT for 15 min. Each euthanized larva was then individually placed in a 1.5 mL microcentrifuge tube, any excess euthanization solution was removed and replaced with 200 µL of TRIzol reagent. Each larva was then homogenized in TRIzol reagent by automated pestle at 7 x 3 sec

bursts. Homogenized larvae were stored at -80 ºC for later use or the protocol was continued as described below. If necessary, samples were thawed on ice within the hood prior to adding 800 µL of TRIzol (total 1 mL TRIzol) then centrifuged at 12,000 *x g* for 15 min. The supernatant was then transferred to a screw-tap tube. Within the screw-cap tube, an additional 1 mL of 95 % ethanol was added and vortexed. This solution was then transferred from the screw-cap tube to a Zymo-Spin IIC Column in a collection tube prior to being centrifuged at 12,000 x *g* for 1 min. Next, the solution collected within the collection tube (flow-through) was discarded and 400 µL of RNA Wash Buffer was added to the same spin column before being centrifuged at 12,000 x *g* for 1 min. The flow-through was once again discarded and 80 µL of DNAse I cocktail (*i.e.* 5 µL DNAse I, 8 µL 10X DNase I Reaction buffer, and 67 µL of DNase/RNase-Free water) was added to the same spin column which was incubated at RT for 15 min before being centrifuged at 12,000 x *g* for 30 sec. Without discarding the flow-through, 400 µL Direct-zol RNA PreWash was added to the same spin column before being centrifuged at 12,000 x *g* for 1 min. The flow-through was discarded before adding 700 µL of RNA Wash Buffer to the same spin column which was then centrifuged at 12,000 x *g* for 1 min. The previous step was repeated twice (*i.e.* three treatments of RNA Wash Buffer in total) before the spin-column was transferred to the final RNase-free tube. Finally, 50 µL of DNase/RNase-free water was added to the column matrix before being centrifuged at max speed for 1 min. The spin column was discarded and the eluted RNA was quantified then either stored at -80 ºC or used immediately for cDNA preparation. RNA stocks were quantified via a pre-programmed ND1000 Nanodrop Nucleic Acid Program. The nanodrop device was blanked with 2 µL of nuclease free water before quantifying 2 µL of each single-fish

RNA stock. RNA stocks with 260/280 values of ~1.8-2, 260/230 values of ~2-2.2, and total concentrations of 60 ng/µL were deemed acceptable for further cDNA preparation.

cDNA Preparation

Following RNA quantification, stored samples were either thawed on ice or directly added to a reaction recipe (*i.e.* 4 µL of 5X reaction buffer, 500 ng RNA stock, made up to a total volume of 20 µL using nuclease free water). The volume of RNA stock necessary to make 500 ng was determined by dividing 500 ng by the RNA concentration determined in the previous section. The volume of nuclease free water necessary for the recipe was determined by subtracting the volume of RNA stock being added from 15 mL. The complete reaction recipe was moved to thermocycler tubes before being treated by a standard thermocycler program. Directly after the thermocycler program was completed, 40 µL of nuclease-free water was added into each reaction recipe-containing thermocycler tube and either stored at -20 ºC or used immediately for qPCR analysis.

qPCR

BioRad SSOSyber Green Supermix (BioRad, #170-8880).was used to perform all qPCR analysis. Following cDNA preparation, stored samples were either thawed on ice or added directly to the qPCR reaction recipe (*i.e.* 5 µL of Sybr Green, 0.15 µL of forward primer, 0.15 µL of reverse primer, 1.7 µL of nuclease free water, and 3 µL of 3:1

diluted cDNA). For large sample sizes, a master mix of Sybr Green, forward primer, reverse primer, and nuclease free water was added to each qPCR plate via a multi-channel pipettor before adding each diluted cDNA sample individually to each well. Each cDNA sample was assessed in duplicate with a no-DNA template included for each gene being assessed. Each qPCR plate was covered with a clear plastic seal before being centrifuged at 5,000 rpm for ~2 sec. The qPCR plate was then treated by the pre-programmed standard thermocycler program.

XI. Zebrafish Sleep Coding

Downloaded EthoVision XT data exported to Excel was first parsed to only include Time (s) and Velocity (cm/s) before being further reduced into 1 s bins (standard EthoVision XT outputs are in 1/30th sec bins). The threshold value for immobility of 0.5 cm/s was then implemented by the equation =IF(B:B>0.5,1,0), where column B indicates the velocity column, such that any 1-sec velocity output greater than 0.5 cm/s was coded as a 1, or mobile, and any 1-sec velocity output less than 0.5 cm/s was coded as a 0. After six consecutive 0s in the binary code, the seventh 0 and any after were coded as sleep. Any 1 that interrupted a string of 0s, or sleep bout, was then coded as the start of a wake bout. The total sleep duration was determined using the equation =COUNTIF(D:D, “S”), where column D indicates the sleep coding column of “S” or “W” for sleep versus wake states. The number of sleep bouts was determined by counting the number of strings of “S” in column D. For ease of counting sleep bouts, all wake bouts were filtered and

highlighted in green and all sleep bouts were filtered and highlighted in purple. To calculate the mean sleep bout length, the total sleep duration was divided by the number of sleep bouts. To calculate the fragmentation index, the number of sleep bouts was divided by the total sleep duration. The Sleep % was determined by dividing the total sleep duration by the sum of the total wake duration and total sleep duration. Total wake duration was determined using the equation =COUNTIF(D:D,"W"). The number of wake bouts was determined by counting the number of strings of "W" in column D. The mean wake bout length was calculated by dividing the total wake duration by the number of wake bouts. The Wake % was calculated by dividing the total wake duration by the sum of the total wake duration and total sleep duration. All sleep variables were converted to minute units before conducting statistical analyses.

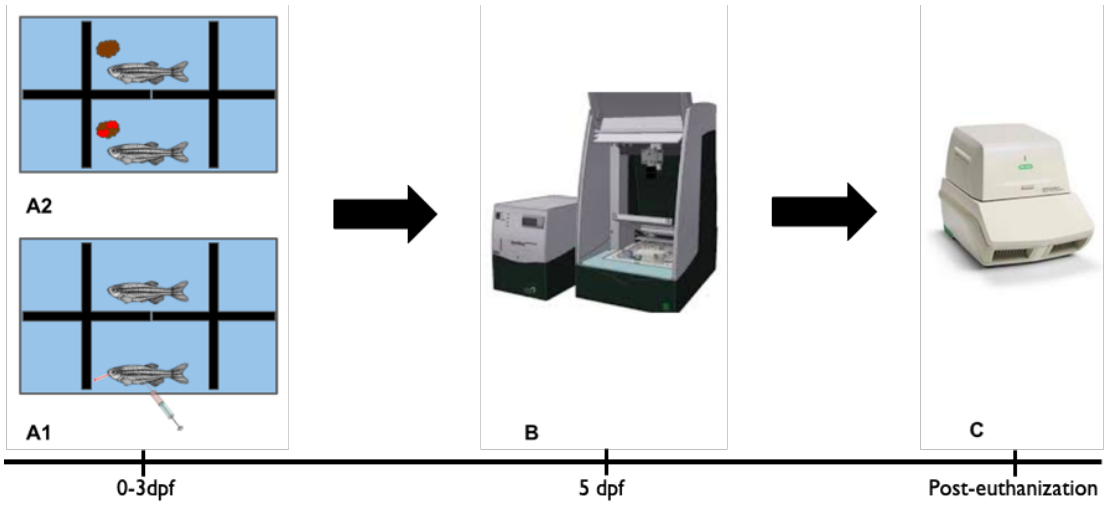

Figure 2. Schematic of the experimental workflow for zebrafish larvae reverse translational analysis. (A1) Infected group larvae were injected with *C. albicans* SN250 culture or mock PBS solution at 1 dpf. **(A2)** Dietary group larvae were fed high-fat diet or typical commercial flake food starting at 3 dpf. **(B)** All zebrafish larvae were subjected to 35 min sleep analysis intervals at 5 dpf. **(C)** All zebrafish larvae were euthanized, homogenized, and used for single fish qPCR immediately after sleep analysis.

Table 2. Translational experimental components for human participants and zebrafish larvae.

	Dietary Analysis	**Sleep Analysis**	**Inflammasome Analysis**
Humans	Dietary intake was not controlled in human participants. Experimental groups (*i.e.* Fat and sugar intake within recommended levels, Fat and sugar intake above recommended levels) were determined using the Dietary Fat and Free Sugar - Short Questionnaire.	All human participants were administered a 7 day home sleep study. All participants slept on the UMaine lab teams sleep recording system for 2 nights. All participants also wore an actigraphy watch for 7 days to serve as a positive control for the novel at-home sleep system.	Human inflammasome gene expression was not analyzed during the 2019 at-home sleep study.
Zebrafish	Zebrafish larvae dietary intake was controlled. Zebrafish were placed into control groups and high fat groups starting at 3 dpf. Control groups were fed typical commercial flake food. High fat groups were fed typical commercial flake food supplemented with 100% cocoa butter.	All zebrafish larvae were subjected to a 35 minute interval sleep analysis utilizing a Noldus DanioVision device at 5 dpf.	To assess the hypothesis that diet-induced gut dysbiosis induced poor sleep quality mediated by inflammasome gene expression, each zebrafish larvae was used for single fish qPCR following Noldus DanioVision sleep analysis.

XII. Zebrafish Statistical Analysis

For statistical analysis, calculations were conducted in Excel (Microsoft Corporation, 2018), R (R Foundation for Statistical Computing, Vienna, Austria, 2017), or GraphPad Prism (La Jolla, CA, USA, 2020) using Shapiro-Wilke test to confirm normality and Student's t-test to assess for variation between experimental and control groups. For qPCR analysis, the ΔΔCt value of each group (*e.g.* control vs. infected, healthy diet vs. high fat diet) was compared using Mann-Whitney test. All plots represent mean +/- standard deviation. For all figures, *$p<0.05$. **$p<0.01$. ***$p<0.001$. ****$p<0.0001$.

RESULTS

I. At-Home Sleep Study Demographics

Table 3. Demographics of nutrient intake within recommended levels and high fat/sugar intake groups

Variable (μ±SE or % (n))	Fat/sugar intake within recommended levels (n = 8)	High fat/sugar intake (n = 5)	p-value
Age, y	71.31±0.91	74.28±0.95	n.s.
Female	77.78%	100.00%	n.s.
Years of education	15.60±0.35	15.34±0.45	0.069
Montreal Cognitive Assessment (MoCA)	26.73±0.30	21.54±0.45	n.s.
Lifestyle factors			
Drinking alcohol	62.2%	56.3%	n.s.
Current or former smoker	46.7%	54.2%	0.071
Current use of sleep medication[a]	42.2%	41.7%	n.s.
Body mass index (BMI)	27.60±0.86	28.03±0.92	n.s.
Obstructive sleep apnea (OSA)	17.8%	28.0%	n.s.
Diabetes	11.1%	20.8%	n.s.
Heart attack or cardiac arrest	11.1%	14.6%	n.s.
Cardiovascular disease	13.3%	18.8%	n.s.
Cerebrovascular disease	6.7%	16.7%	n.s.
Traumatic brain injury (TBI)	15.6%	18.8%	n.s.
Hypercholesterolemia	42.2%	41.7%	n.s.
Hypertension	34.1%	47.9%	n.s.
Arthritis	47.7%	55.4%	n.s.
Thyroid disease	33.3 %	25.0%	n.s.
Current depressed mood[a]	13.3%	37.5%	n.s.

Note: Values are shown as % or mean ± SE, and compared by independent samples t-test (continuous variables) or chi-square test of association (categorical variables).

[a] Current use of sleep medication was described as any self-reported use of over the counter or prescribed sleep medication over the last month

[b] Current depression was indicated by self-report answering the question "Are you currently feeling depressed?"

In regards to the participant's initial demographic screening, a trend for an association between years of education and fat/sugar intake within recommended levels was observed. Furthermore, a trend for being a current or former smoker and high fat/sugar intake was observed. Finally, it should be noted that all of the participants enrolled in my pilot study who were placed into the high fat/sugar intake group were female. While these demographic characteristics are not directly applicable to the hypothesis of my study, they do provide notable insight into the lifestyle choices and social determinants of health pertaining to the participants enrolled in this pilot study.

II. Dietary Fat and Free Sugar - Short Questionnaire Self Reported Values

Table 4. Saturated fat and free sugar intake scores according to the Dietary Fat and Free Sugar - Short Questionnaire

Participant ID	Saturated fat and free sugar intake scores
096	59
097	71*
098	47
099	36
100	23
101	66*
102	50
103	50
104	78*
105	50
106	70*
107	74*
109	51

Note. *Participant's saturated fat and free sugar intake likely exceeds recommended levels.

Sleep fragmentation is both a risk factor for and a symptom of Mild Cognitive Impairment, the clinical prodrome of Alzheimer's Disease. As such, the development of early identification diagnostic tools for sleep fragmentation and other disrupted sleep patterns could help in preventing the progression of MCI into AD, an agenda which is of particular importance for Maine's aging population. Starting in 2015, the Hayes Lab conducted a study which optimized a sensor-embedded mattress device capable of diagnosing MCI through one's sleep quality within a participant's home environment. The objective of this device was to provide a more accessible and affordable option for aging individuals to assess their sleep quality, especially for those who had family history of sleep disorders and age-related neurodegenerative disorders, in order to mitigate one's risk of developing MCI, AD, and related forms of dementia. As is the case with many lifestyle factors, sleep can be impacted by countless factors including dietary choices. For this reason, the nutritional profile of each participant enrolled in the at-home sleep study was assessed by administering the Dietary Fat and Free Sugar - Short Questionnaire (DFS) in order to analyze the power of diet as a moderator of sleep quality and related neuropathology. The DFS is a 26 question self-reported survey in which individuals are asked to self-rank the frequency of how often they consume certain foods, particularly those high in fat and sugar content. Each question is scored 1-5 and the overall survey is summated. Those who score ≥60 or greater on the DFS are likely consuming a diet of fat and sugar above the WHO's recommended levels.

Within the context of the ongoing clinical study, each participant was administered the DFS on Day 1, prior to any sleep analysis. The results of the total summated DFS scores for all participants (n=13) are shown in Table 2. Five of the

thirteen participants (38.46%) scored equal to or greater than 60 on the DFS, indicating that their fat and free sugar intake was above the WHO's recommended levels. These five participants were placed into the 'High fat/sugar intake' group while the remaining eight participants were placed into the 'Fat/sugar intake within recommended levels group'. At the end of the one week study, these two groups were assessed as potential moderators of each sleep variable recorded by the novel sleep recording device. As indicated in Table 3 below, Independent sample T-tests were run in order to analyze the association between fat/sugar intake and actigraphy output. The sleep metrics described in this pilot study were interpreted by actigraph, an accompanying measure to the novel sleep recording device (1014). Sleep time was different between groups and strongly increased in the diet risk group. Group findings show that "Fat/sugar intake within recommended levels" (M=5.78, SD=2.45) and "High fat/sugar intake" (M=14.79, SD=4.62) conditions were statistically different, $t(11)=-4.66$, $p = 0.001$, revealing greater sleep duration in the High fat/sugar intake group. There was a trend for the association in the scores for immobility during sleep measured by the mean number of immobile phases between the "Fat/sugar intake within recommended levels"group (M=37.13, SD=8.06) and "High fat/sugar intake"group (M=52.44, SD=20.41) conditions; t(11=-2.01, $p = 0.070$). As shown in Table 4, actigraphy findings suggest that poor diet increased sleep duration, a companion marker of sleep loss, and disrupted motor activity during sleep, measured by more transitions from mobile to immobile activity levels, which is consistent with increased sleep to wake state events and sleep fragmentation.

III. Human Participant Sleep Variables

Table 5. Actigraphy variables for fat/sugar intake within recommended levels and high fat/sugar intake groups

Variable	Fat/sugar intake within recommended levels (n=8)	High fat/sugar intake (n=5)	*p*-value
Mean Time in Bed	519.67±28.70	524.72± 47.50	n.s.
Mean Assumed Sleep	499.77±26.81	489.86±45.91	n.s.
Mean Actual Sleep Time	443.79±30.16	420.21±42.88	n.s.
Mean Actual Sleep %	88.59±1.92	85.90±3.77	n.s.
Mean Actual Wake Time	55.76±7.37	69.54±18.46	n.s.
Mean Actual Wake %	11.41±1.92	14.12±3.77	n.s.
Mean Sleep Efficiency	85.17±1.77	79.85± 2.72	n.s.
Mean Sleep Latency	14.12±4.19	20.07±4.79	n.s.
Mean Sleep Bouts	21.71±2.24	26.97±4.83	n.s.
Mean Wake Bouts	21.15±2.20	26.50±4.90	n.s.
Mean Mean Sleep Bout Time	23.84±3.27	17.84±3.12	n.s.
Mean Mean Wake Bout Time	2.68±0.36	2.68±0.65	n.s.
Mean Number of Minutes Immobile	428.41±27.69	384.25±41.77	n.s.
Mean Immobile % Time	85.68±1.89	78.76±5.73	n.s.
Mean Number of Minutes Moving	71.29±7.92	105.57±27.84	n.s.
Mean Moving % Time	14.32±1.89	21.19±5.76	n.s.
Mean Number of Immobile Phases	37.13±2.69	52.44±10.21	0.070

Table 5 continued

Mean Mean Length Immobility	12.47±1.07	9.16±2.47	n.s.
Mean One Minute Immobility	7.44±1.25	13.29±3.96	n.s.
Mean One Minute Immobility %	18.16±1.84	22.62±5.69	n.s.
Mean Total Activity Score	8282.05±1418.17	9445.93±2764.52	n.s.
Mean Fragmentation Index	32.48±3.74	43.85±11.40	n.s.
Sleep Time	5.78±0.82	14.79±2.31	**0.001**

Note. x ± y represents the mean ± standard error of the mean (SEM), (n) = sample size.
Note. x ± y represents the mean ± standard error of the mean (SEM), (n) = sample size.

The results reported of the at-home sleep study stimulated the idea for a reverse-translational follow-up study which aimed to further elucidate the putative molecular and physiological mechanism connecting Western Diet to perturbed sleep quality. It was hypothesized that Western Diet, a known inducer of gut dysbiosis and systemic inflammation, triggered pro-inflammatory signaling pathways involving pyrogenic and somnogenic cytokines which caused the observable sleep disruption. In order to assess the above hypothesis, zebrafish were selected as a model organism given that they have comparable sleep ontogeny to humans as well as a highly homologous immune system. It was hypothesized that zebrafish who were experiencing systemic inflammation would produce greater immune gene expression of inflammatory cytokines with both pyrogenic and somnogenic effects. In addition to this, it was hypothesized that such differential cytokine gene expression would serve as a moderator for perturbed sleep quality in zebrafish larvae. Inflammation was induced in zebrafish larvae by direct infection with human fungal pathogen *Candida*, meant to serve as a strong inflammatory response

positive control, or by a high fat diet, meant to serve as an experimental model for the Western Diet consumed by human participants in the initial sleep study. Respective controls for each method of inflammation were also incorporated (*i.e.* half of the infected group was injected with a mock-PBS solution and half of the dietary group was fed normal commercial flake food). Following inflammation induction, as elicited via pathogen infection or a high fat diet, each zebrafish larva was subjected to a 35 minute sleep analysis within the last hour of their 14 hour dark cycle and subsequently euthanized for single fish qPCR analysis. This overall experimental workflow was repeated such that the 6 replicate sleep analysis trials and 2 replicate qPCR analyses were conducted for the infected group and 4 replicate sleep trials and 2 replicate qPCR analyses were conducted for the dietary group. However, only 1 of the sleep trials performed for the dietary group was used for data analysis due to experimental error involving file export within the Noldus EthoVision XT program. The results of both the infected zebrafish group and the high fat diet zebrafish group are discussed extensively below.

IV. Infected Zebrafish Sleep Analysis

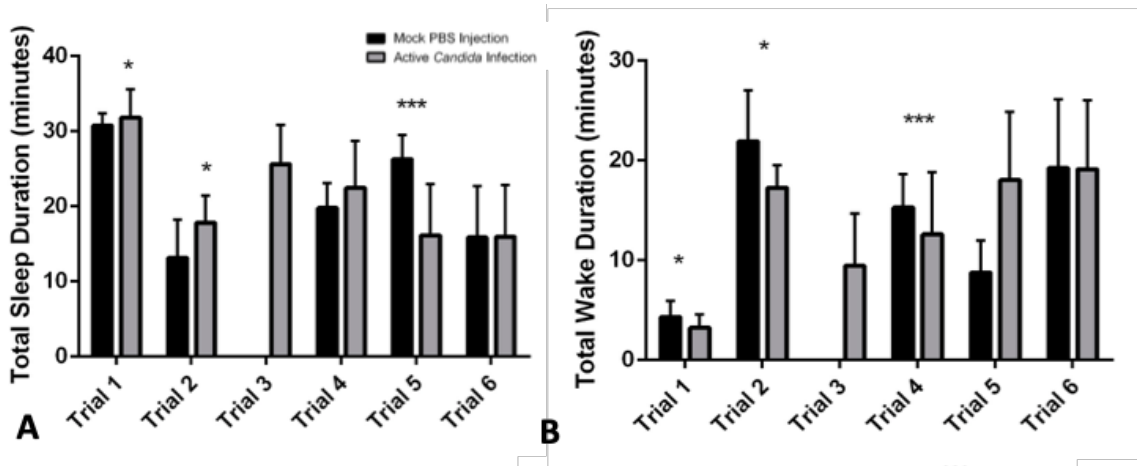

Figure 3. Total sleep and wake duration (minutes) and sleep and wake % for Mock PBS Injected and Active *Candida* Infection zebrafish larvae in repeat sleep analysis Trials 1-6. (A) Total Sleep Duration of control and infected zebrafish. **(B)** Total wake duration of control and infected zebrafish larvae. Though not shown here, sleep and wake % depicted identical results to total sleep and wake duration. Bar plots and error bars represent Mean and SD. *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.

All sleep variables analyzed in zebrafish larvae were selected on previous optimization and proof-of-concept analyses as well as reverse-translational power to human actigraphy variables. Zebrafish larvae sleep analyses were conducted at 35 min intervals within the last hour of the assigned 14-hour dark cycle. Zebrafish sleep analysis is distinct from humans in that measures pertaining to time spent 'in bed' (*e.g.* Sleep Latency) cannot accurately be assessed. However, total sleep and wake duration and percentage (Figure 3) as well as the number of sleep and wake bouts, mean length of

sleep and wake bouts, and fragmentation indexes (Figure 4) are mutual sleep variables which have been reported in both humans and zebrafish. Thus, in the place of actigraphy sensors, zebrafish immobility and velocity states can be used to predict sleep and wakefulness as well as the transition between these two states. As observed in Figure 3 above, sleep variables are highly irregular between identical repeat experiments as well as within some experimental groups. This observation could serve to explain the absence of a statistically significant difference between each sleep variable in the Active *Candida* Infection group (n=56) and the Mock PBS Injection group (n=55) data sets (Appendix C). However, statistically significant observations can still be used to assess trending results between experiments.

For all zebrafish sleep variables, sample normality was assessed by Shapiro-Wilk test and independent variables were assessed by Student's t-test. In 80% of infected zebrafish sleep analyses, the Active *Candida* Infection group exhibited a greater total sleep duration and sleep %, and subsequently a lower Total Wake Duration and Wake %, than the Mock PBS Injection group. Moreover, Trials 1, 2, and 5 exhibited statistically significant variation in these sleep parameters (Trial 1: n=20, p=0.01831.Trial 2: n=22, p=0.012241. Trial 5: n=22, p=0.000173).

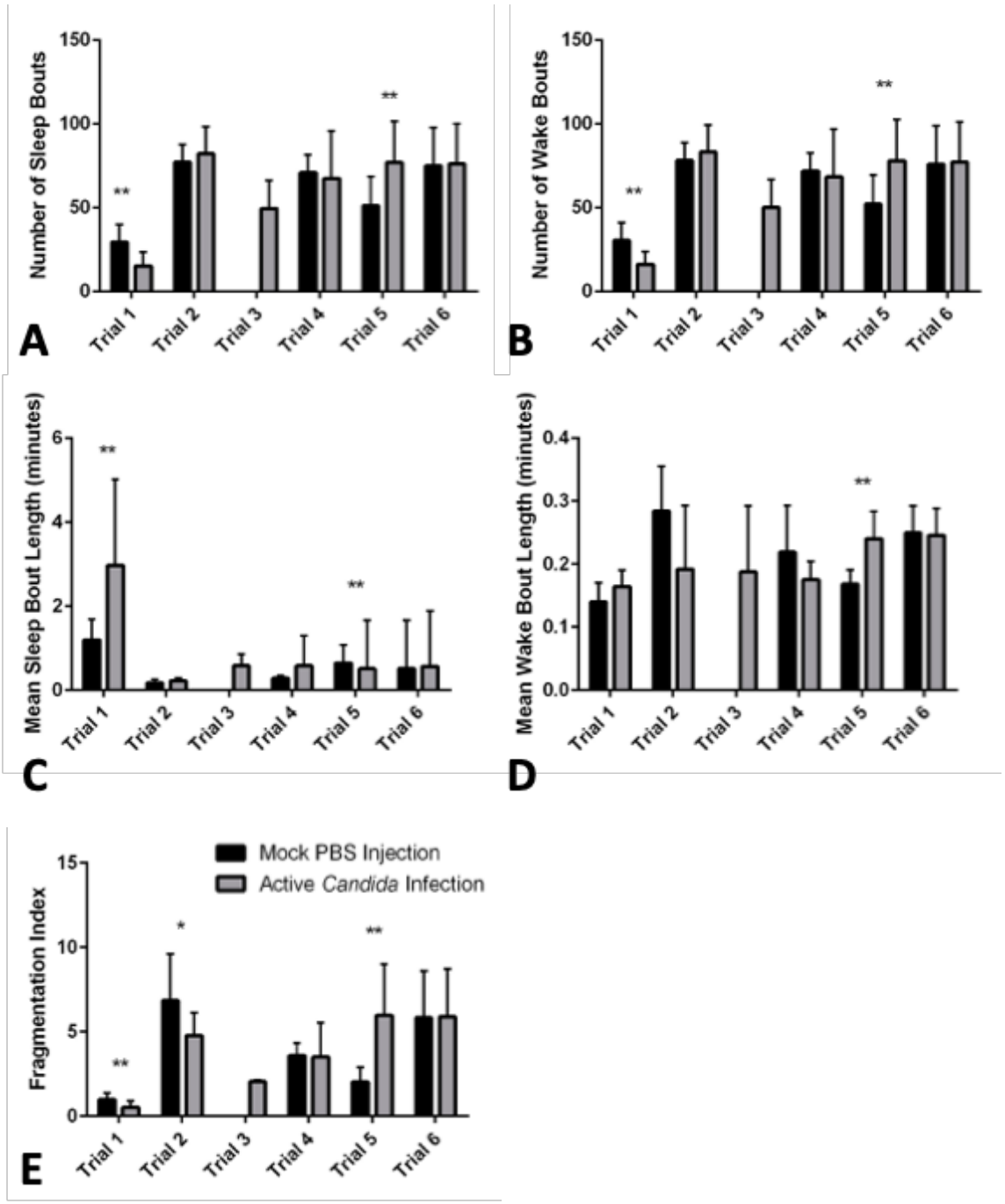

Figure 4. Number of sleep and wake bouts, mean sleep and wake bout length (minutes), and fragmentation indexes for PBS injected and active *Candida* infection zebrafish larvae in repeat sleep analysis Trials 1-6. (A) Total Sleep Duration of control and infected zebrafish. **(B)** Total wake duration of control and infected zebrafish larvae.

(C) Sleep % of control and infected zebrafish larvae. **(D)** Wake % of control and zebrafish larvae. Bar plots and error bars represent Mean and SD. *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.

In 60% of infected zebrafish sleep analyses the Active *Candida* Infection group exhibited a greater number of sleep bouts than the Mock PBS Injection though only Trial 5 yielded statistically significant results (Trial 5: n=22, p=0.00135). Similarly, in 80% of infected zebrafish sleep analysis the Active *Candida* Infection group exhibited a greater mean sleep bout length than the Mock PBS Injection group though only the results of Trial 1 were statistically significant (Trial 1: n=20, p=0.005955). Regardless of sleep bout length, by definition the end of each sleep bout must simultaneously signal the beginning of a wake bout. Thus, larvae who had a higher Number of Sleep Bouts also yielded a higher number of wakebBouts. While this may not appear to add obvious information for analysis, wake variables are an integral part of human sleep analysis and, thus, should be included for zebrafish sleep studies to maintain reverse translational power. Such characteristic brief, intermittent arousals in zebrafish larval sleep was further quantified by the fragmentation index. Specifically, in 60% of infected zebrafish sleep analyses the Active *Candida* Infection group exhibited a lower fragmentation index than the Mock PBS Injection group with both Trials 1 and 2 yielding statistically significant results (Trial 1: n=20, p=0.009936, Trial 2: n=22, p=0.021788). The results reported in Figures 3 and 4 indicate that zebrafish experiencing inflammation induced via direct pathogen infection tend to have more frequent sleep bouts (*i.e.* periods of immobility), longer sleep bouts, and overall exhibit greater sleep duration than zebrafish who are not infected.

V. High Fat Diet Zebrafish Sleep Analysis

The results of the infected zebrafish sleep analysis are not inconsistent with the hypothesis that zebrafish larvae experiencing strong inflammation induced by fungal pathogen *Candida* exhibit perturbed sleep quality, particularly in regards to sleep duration and the number and length of sleep bouts (Figures 3, 4). As such, given that the infected zebrafish group was meant to serve as a positive control for the putative inflammatory pathways hypothesized to be triggered by a high fat diet, further experimentation was done to assess the power of a high fat diet as a moderator of sleep quality in zebrafish.

Beginning at 3dpf, following manual dechorionation at 2 dpf and expected development of a functioning digestive tract occurring as early as 3 dpf, zebrafish larvae were split into 'Control Diet' and 'High Fat Diet' groups. The Control Diet was prepared by suspending 1 g of Gemma 75 commercial flake food (Skretting Zebrafish, 2021.) in 100 mL of deionized water. The High Fat Diet was prepared under similar conditions but was supplemented with 10% (w/w) pure cocoa butter mixed via heated stirring. Both feeds were autoclaved and allowed to cool at room temperature prior to feeding in order to mitigate the risk of bacterial colonization of the feed prior to consumption as this would induce non-high fat diet-mediated gut microflora shift and potentially inflammatory signaling. Identical statistical analyses were performed on the high fat zebrafish group. As was observed in the infected zebrafish group, diet-controlled zebrafish displayed highly variable sleep metrics between identical repeat experiments. The results of the high fat zebrafish sleep analysis are discussed extensively below.

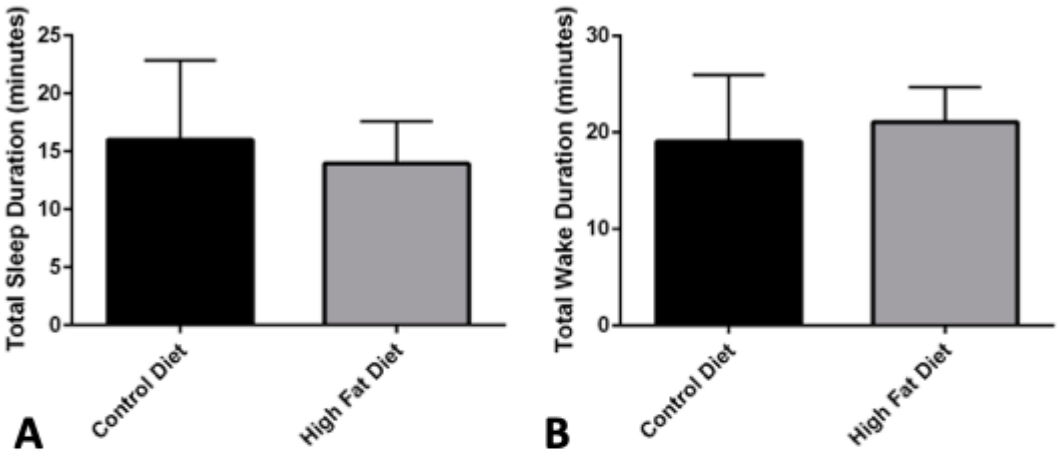

Figure 5. Total sleep and wake duration (minutes) in control diet and high fat diet zebrafish larvae in repeat sleep analysis Trials 1-6. (A) Total Sleep Duration of control and high fat fed zebrafish. **(B)** Total wake duration of control and high fat fed zebrafish larvae. Though not shown, sleep and wake % depict identical results to total sleep and wake duration. *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.

Though no values were found to be statistically significant, the results in Figure 5 report that High Fat fed zebrafish generally exhibited depressed total sleep duration and sleep % and subsequently a greater total wake Duration and Wake %. Though any lack of statistical significance could be due to uneven control and experimental groups as well as a small sample size. Similarly, as shown in Figure 6 below, high fat fed zebrafish had a greater number of sleep bouts and shorter mean sleep bout lengths than control diet fed zebrafish. Furthermore, high fat fed zebrafish exhibited a greater fragmentation index than control diet fed zebrafish. Future research should repeat this experiment to produce a larger sample size in order to account for natural variability in zebrafish larvae sleep analyses and to highlight any potentially unobserved trends in differential sleep quality.

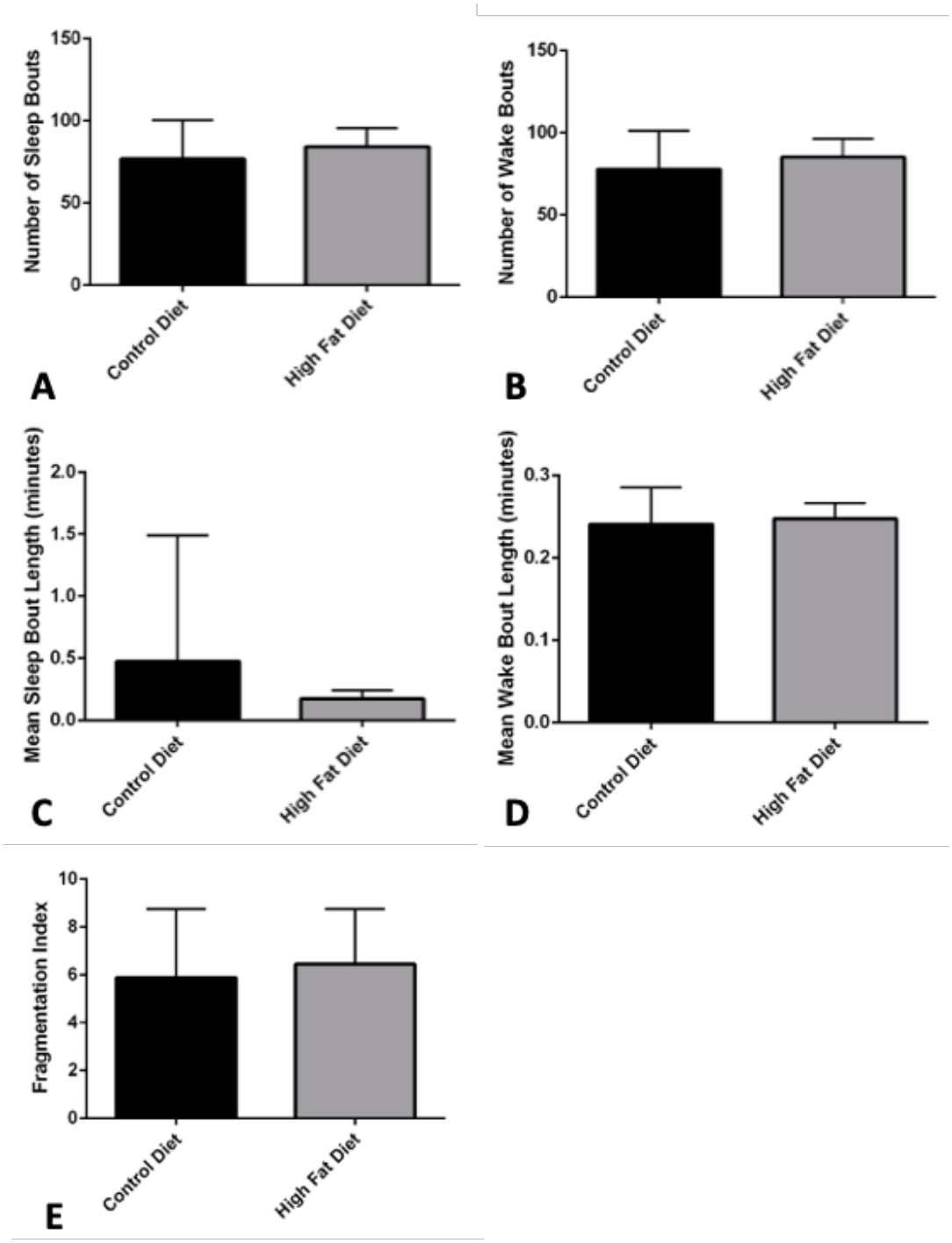

Figure 6. Number of sleep and wake bouts, mean sleep and wake bout length (minutes), and fragmentation indexes for control diet and high fat diet zebrafish

larvae in repeat sleep analysis Trials 1-6. (A) Total sleep duration of control and high fat fed zebrafish. **(B)** Total wake duration of control and high fat zebrafish larvae. **(C)** Sleep % of control and high fat fed zebrafish larvae. **(D)** Wake % of control and high fat fed zebrafish larvae. Bar plots and error bars represent Mean and SD. *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.

While statistically significantly differential sleep quality was observed in *Candida* infected zebrafish larvae, and non-significant differences were observed in high fat fed zebrafish larvae, these associative observations were not sufficient to address the entire aim of this study. Namely, single fish qPCR experimentation was necessary to elucidate if elevated somnogenic and pyrogenic cytokine expression mediated perturbed sleep quality in either group. In order to accomplish this, directly following sleep analysis each larvae was euthanized, homogenized, and stored at -80 ºC for future use in quantitative real time PCR (qPCR).

VI. Infected Zebrafish qPCR Analysis

As with any qPCR experimental design, it was critical that proper target genes were selected for analysis in order to accurately address the hypothesis that pro-inflammatory cytokines served to induce sleep disruption in both *Candida* infected and high fat fed zebrafish. As such, TNF-α, IL-1β, and IL-6 were assessed in both zebrafish experimental groups as, to date, these signaling molecules are the most comprehensively understood cytokines known to regulate *both* the pro-inflammatory response and sleep state behaviors (Kreuger, 1989).

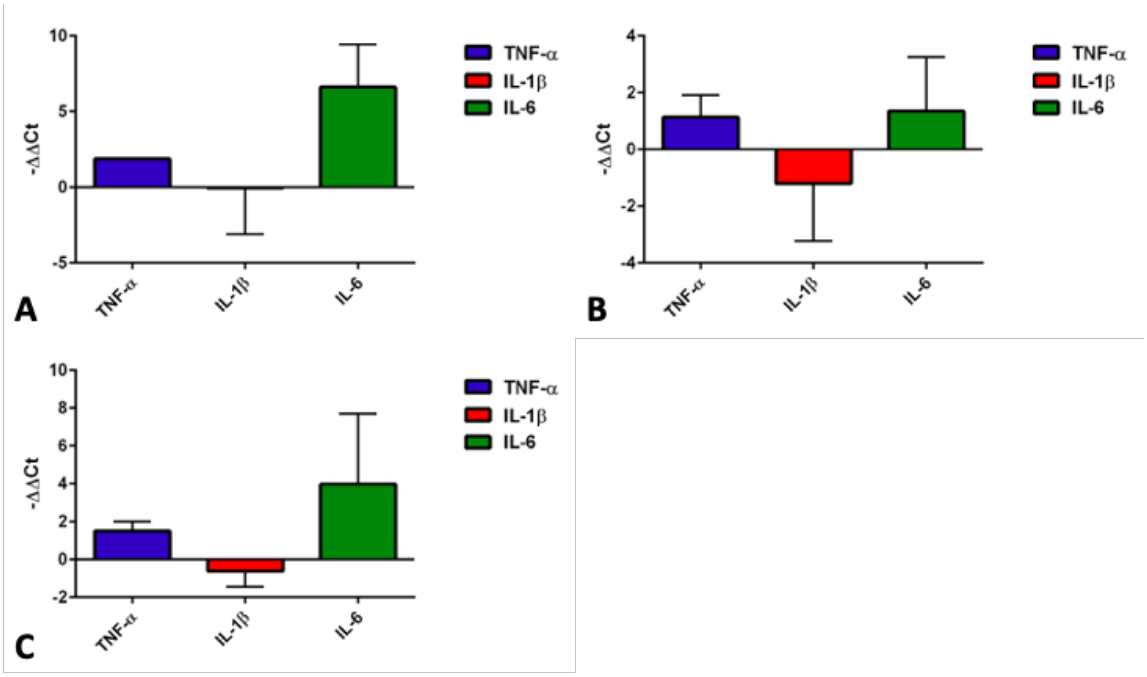

Figure 7. Gene expression of TNF-α (Blue) and IL-6 (Green) is upregulated (n.s.) in *Candida* infected zebrafish larvae. Il-1β (Red) is depressed (n.s.) in *Candida* infected zebrafish larvae. **(A)** qPCR Trial 1. **(B)** qPCR repeat Trial 2. **(C)** Pooled ΔΔCt of Trial 1 and 2. Bar plots and error bars represent Mean and SD. Ct values correspond to initial qPCR Cycle threshold output values. ΔCt values indicate the difference between target and non-target gene expression. ΔΔCt values indicate the difference between single *Candida* infected larvae ΔCt values and the average ΔCt value of control larvae. Thus, -ΔΔCt values represent the direction of fold change in gene expression.

qPCR Real-Time Systems produce cycle threshold (Ct) values which are inversely proportional to the levels of targeted mRNA in the provided sample. Though Ct values serve as necessary intermediates in any qPCR analysis, they do not provide meaningful quantitative data which can easily be interpreted. As such, ΔCt, ΔΔCt, and even fold change expressions are commonly calculated. Of the three, ΔCt values are the

only metric which maintain the identity of a single zebrafish larvae's gene expression. ΔCt values are calculated by subtracting the housekeeping gene (*i.e.* GAPDH) expression of a particular larvae from the target gene expression of a particular larvae. As such, ΔCt values serve to show the difference between target and control, baseline samples. Further, ΔΔCt serves to explain the difference between a treated and untreated sample. ΔΔCt values are calculated by subtracting the average ΔCt of a control larvae from the ΔCt of an individual experimental larvae. In this manner, ΔΔCt values indicate differential expression induced by the experimental moderator. Given that gene expression fold change can be calculated by the negative log base two of ΔΔCt values, a negative ΔΔCt value is indicative of elevated gene expression and a positive ΔΔCt is indicative of depressed gene expression when comparing the treated group to the control group. Or, as seen in Appendix F, infected larvae who exhibit a negative ΔΔCt value for a target cytokine produce fold change values above 1 indicative of elevated gene expression while infected larvae who exhibit a positive ΔΔCt value produce fold change values below 1 indicative of depressed gene expression. In Figure 7 above, it can be seen that in both Trial 1, Trial 2, and the pooled data set for infected larvae zebrafish, TNF-α and IL-6 both have negative ΔΔCt values while IL-1β has a positive ΔΔCt value. Thus, the experimental replicate results suggest that direct *Candida* infection serves to upregulate TNF-α and IL-6 gene expression while down regulating IL-1β expression.

VII. High Fat Diet Zebrafish qPCR Analysis

As discussed above, the strong positive control for pro-inflammatory and somnogenic cytokine release, direct *Candida* infection, was shown to be correlated with perturbed sleep quality *and* elevated expression of cytokines TNF-α and IL-6. These results incited investigation into the hypothesized mechanism of high fat-mediated gut dysbiosis-inducing systemic inflammatory pathways. As such, identical experimental methods were conducted as was described within the infected zebrafish group except ΔΔCt values were calculated in order to identify differential gene fold expression for high fat diet treated zebrafish as compared to control diet fed zebrafish.

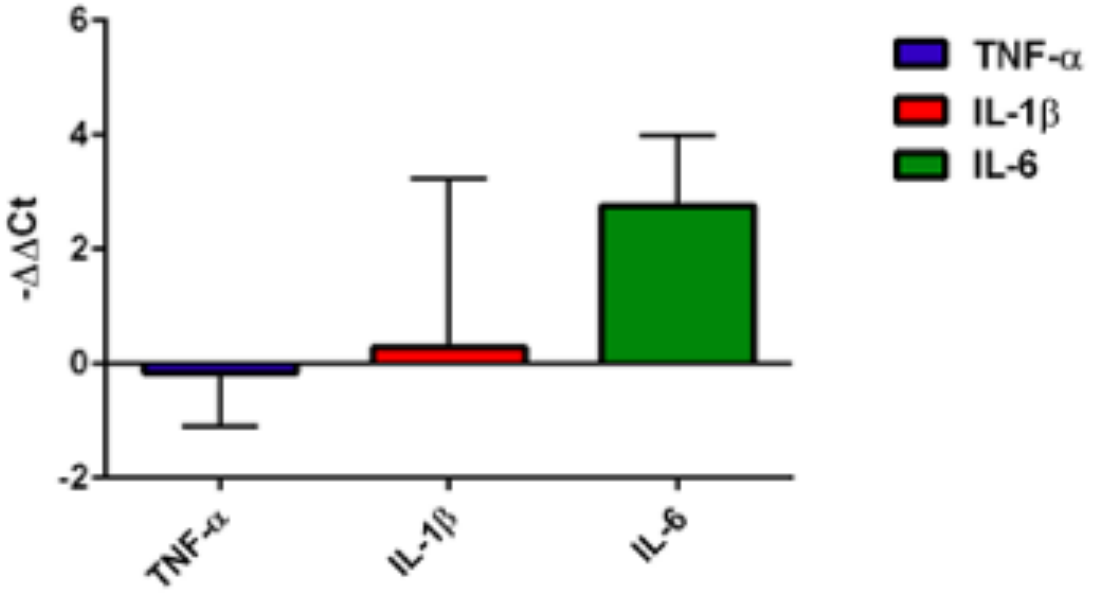

Figure 8. Gene expression of IL-1β (Red) and IL-6 (Green) is upregulated (n.s.) in *Candida* infected zebrafish larvae. TNF-α (Red) is depressed (n.s.) in *Candida* infected zebrafish larvae. Pooled ΔΔCt of high fat diet qPCR replicate experiments Trial 1 and 2 were compiled due to high similarity between samples. Bar plots and error bars represent

Mean and SD. Ct values correspond to initial qPCR Cycle threshold output values. ΔCt values indicate the difference between target and non-target gene expression. ΔΔCt values indicate the difference between single high fat diet treated larvae ΔCt values and the average ΔCt value of control larvae. Thus, -ΔΔCt values represent the direction of fold change in gene expression.

As seen in Figure 8 above, both IL-1β and IL-6 exhibit negative ΔΔCt values when comparing high fat fed zebrafish larvae gene expression to that of control diet zebrafish larvae. However, the differential expression observed in both TNF-α and IL-1β shown above is relatively insignificant, or close to zero, while IL-6 appears to be highly upregulated in comparison. Most notably, IL-6 also showed the most profound negative ΔΔCt value, and therefore the greatest increase in gene fold expression, in the pro-inflammatory positive control group, *Candida* infected zebrafish. Thus, qPCR analysis of both the infected zebrafish larvae and the high fat diet treated zebrafish larvae could provide grounds suggesting that both infection and Western Diet elicit an IL-6 mediated pro-inflammatory response leading to perturbed sleep states. At the same time, however, future research will need to assess the relatively low sample size in both experimental groups in order to compensate for the natural variation of cytokine gene expression between individual zebrafish larvae in order to better elucidate this potential signaling mechanism.

VIII. Correlation Between Pro-inflammatory Cytokine Gene Expression and Specific Zebrafish Sleep Variables

The differentially expressed pro-inflammatory cytokine gene expression observed between control and experimental groups treated with different means of inflammation-inducing mechanisms holds promise for future work in elucidating the physiological phenomena underlying the connection between diet and sleep quality, particularly in regards to the role of IL-6 in modulating these events. As such, exploratory correlation models were conducted to attempt to pinpoint an association between specific elevated cytokine gene expression and distinct sleep variable metrics. The results of these exploratory analyses for both Trial 1 and Trial 2 of the *Candida* infected experimental group are described below. The high fat diet experimental group was not incorporated in these analyses due to low sample size and absence of single fish sleep metrics in both qPCR trials.

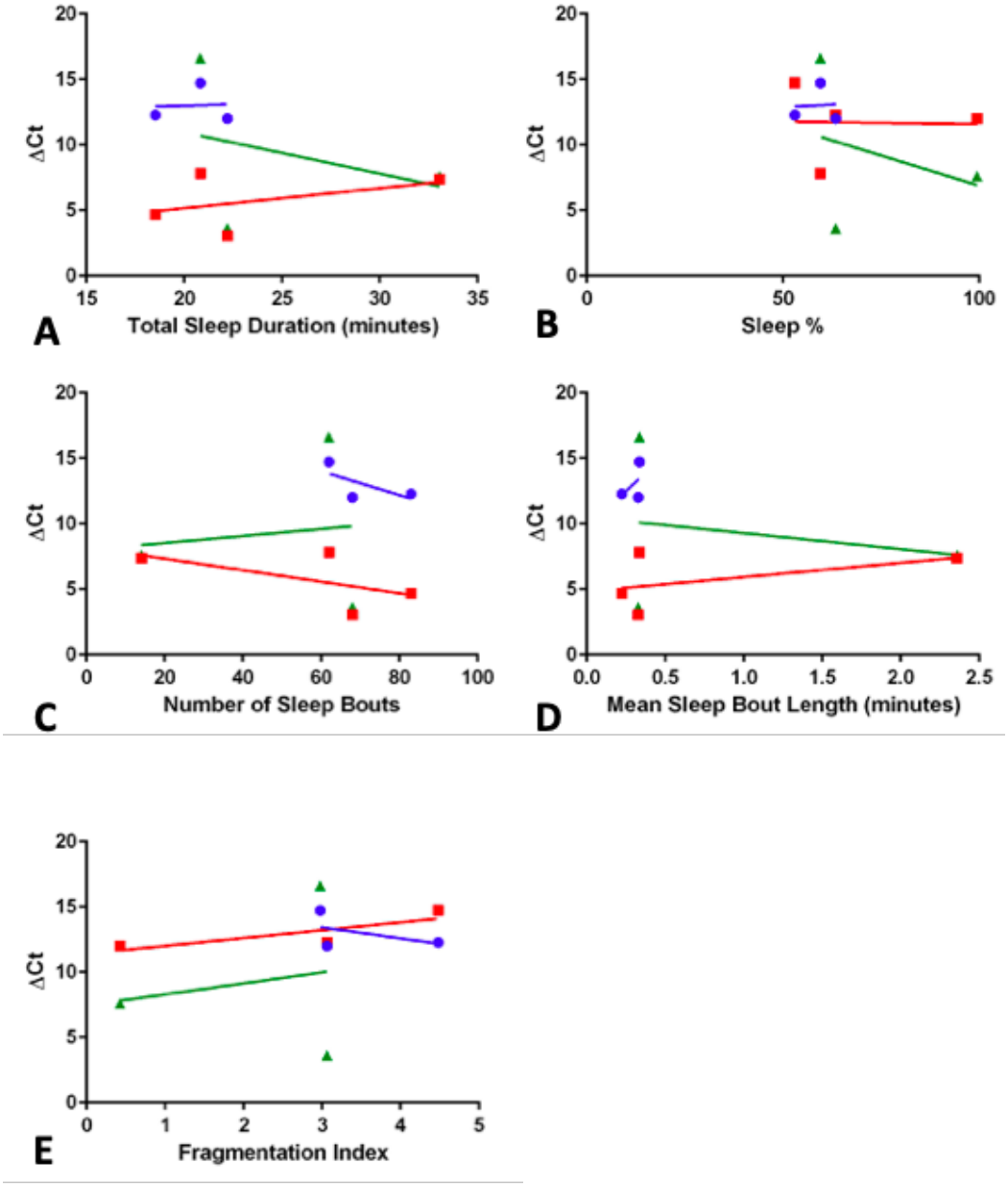

Figure 9. Exploratory linear regression analyses of individual sleep variables and TNF-α (Blue), IL-1β (Red), and IL-6 (Green) ΔCt values for infected zebrafish qPCR Trial 1. (A) Single fish Total Sleep Duration (minutes). **(B)** Single fish sleep %. **(C)** Single fish number of sleep bouts. **(D)** Single fish mean sleep bout length (minutes). **(E)** Single fish fragmentation index. All sleep variables are plotted against the single fish ΔCt. Trend lines represent linear least squares regression lines.

While the ΔΔCt typically provides information necessary for interpreting gene fold expression, it eliminates the quantification of zebrafish at the single larvae level given that it is calculated by subtracting the average ΔCt of the control group from the single fish ΔCt of each experimental larvae. Thus, in order to assess a relationship between each individual fish's inflammatory cytokine expression profile and sleep quality, ΔCt values were plotted as opposed to ΔΔCt values. In these plots, each point represents a single fish with the y-axis indicating its ΔCt value for a given target gene and the x-axis indicating its sleep quality described by the defined variable. As such these plots allow for quantification of any potential relationship between pro-inflammatory cytokine ΔCt values and sleep disruption of single zebrafish larvae. Least squares regression lines were fitted to each cytokine, TNF-α (Blue), IL-1β (Red), and IL-6 (Green), and the R^2 value of each regression line was interpreted to quantify how well each regression line fit the dataset. R^2 values have a range from 0-1 with 1 being a perfect fit to the linear descriptive equation. However, the only R^2 value reporting over 50% (0.5) was that which described the relationship between elevated IL-1β expression and single fish Fragmentation Indexes (R^2=0.6723). Thus, the exploratory analyses of the infected zebrafish larvae Trial 1, as well as the sleep variables of each single fish being plotted, indicate an association between increasing IL-1β expression and elevated sleep fragmentation.

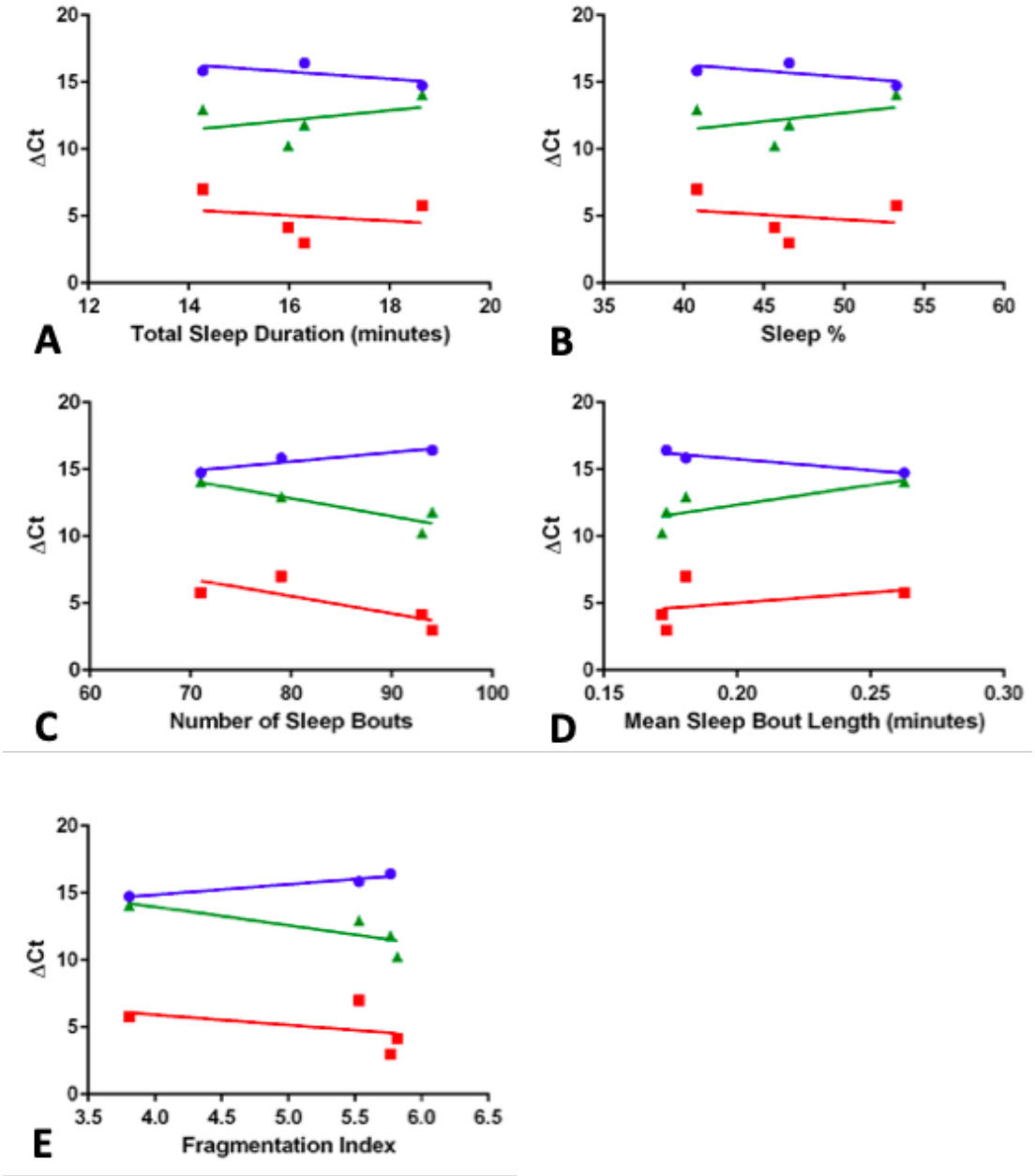

Figure 10. Exploratory linear regression analyses of individual sleep variables and TNF-α (Blue), IL-1β (Red), and IL-6 (Green) ΔCt values for infected zebrafish qPCR Trial 2. (A) Single fish total sleep duration (minutes). **(B)** Single fish Sleep %. **(C)** Single fish number of sleep bouts. **(D)** Single fish mean sleep bout length (minutes). **(E)** Single fish fragmentation index. All sleep variables are plotted against the single fish ΔCt. Trend lines represent linear least squares regression lines.

Identical plotting methods were conducted for infected zebrafish larvae qPCR Trial 2. As seen in the scatterplots above(Figure 10) and numerically in Appendix H, the ΔCt values of qPCR Trial 2 were much tighter than those of Trial 1. Thus, more information could be determined from plotting the results of the qPCR experiment against the single fish sleep variables discussed previously. Again, linear least squares regression lines were calculated with any linear regression reporting an R^2 value over 0.5 being deemed an acceptable 'fit'. A trend for the correlation was observed between inflammatory cytokine ΔCt values and individual sleep variables in infected zebrafish larvae. For one, a correlation between increased TNF-α expression (R^2=0.8819) as well as increased IL-6 expression (R^2=0.8212) and an increase in the number of sleep bouts was observed. Further, a correlation between increased TNF-α expression (R^2=0.9279) and elevated mean sleep bout length was observed. Finally, a correlation between increased IL-6 expression (R^2=0.6567) and greater Fragmentation Indexes was observed. As such, the results of

DISCUSSION

Clinical implications of sleep disturbances are becoming increasingly apparent thereby increasing the demand for research aimed at elucidating the physiological mechanisms which relate disease states and sleep disruption. Further, a more robust understanding of how basic lifestyle factors, such as diet, influence sleep quality is a critical component of such research. Preliminary results reported by Hayes suggested that aging adults who consumed a diet higher in fat and free sugar exhibited poorer sleep quality, particularly in regards to sleep duration-related metrics and sleep fragmentation. In line with previous work which has found that Western diet induces gut-dysbiosis as well as elevated pro-inflammatory cytokine expression it was subsequently hypothesized that aging individuals who consumed a more severe Western diet experienced pro-inflammatory signaling known to contribute to sleep disruption[80,81]. To assess parts of this hypothesized mechanism of causation, a reverse-translational protocol was optimized to attempt to model this phenomenon in zebrafish. Zebrafish larvae (≤5 dpf) were administered a high fat or control diet beginning at 3 dpf before being subjected to a 35 minute sleep analysis at 5 dpf. Another experimental group of zebrafish was not controlled by diet but instead received direct infection of human fungal pathogen, *Candida*, or a mock infection. Both were intended to mimic the hypothesized inflammatory state induced by the aforementioned high fat diet. Following sleep analysis, each larvae was used for single fish qPCR to measure pyrogenic cytokine gene expression. The pro-inflammatory cytokines selected (*i.e.* TNF-α, IL-1β, and IL-6) were selected as they are also known to have somnogenic properties[23]. Thus, cytokine gene expression was analyzed separately from sleep quality to attempt to identify first if sleep

disruption was observed and subsequently if any perturbed sleep quality was correlated with elevated pro-inflammatory gene expression.

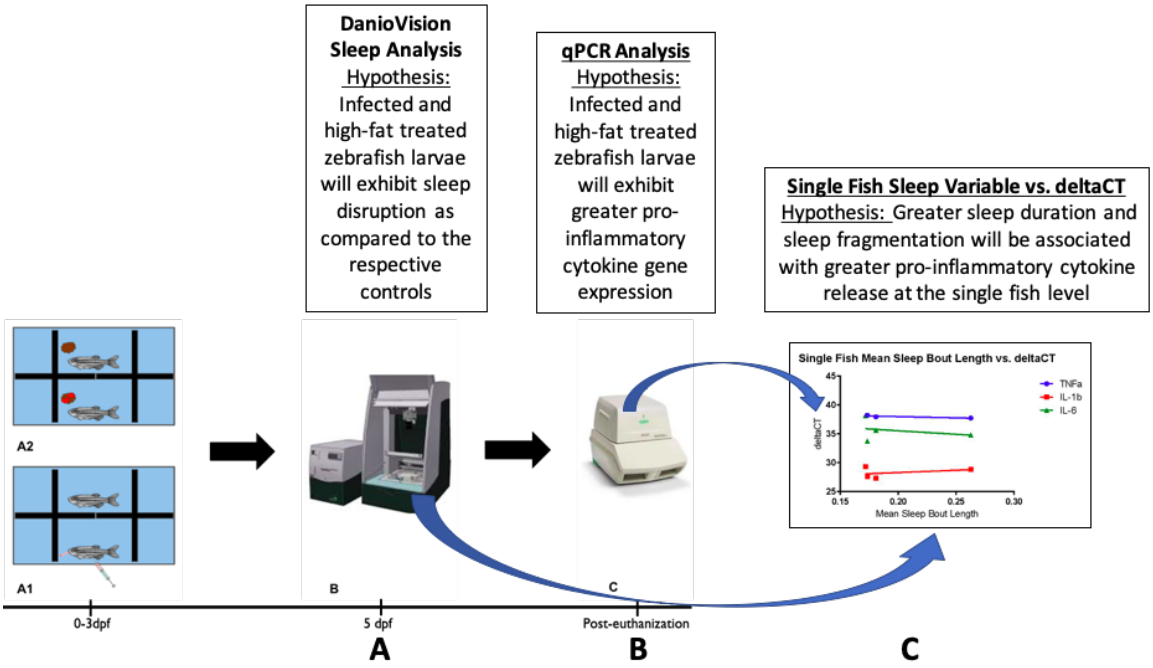

Figure 11. Schematic representation of how each component of the reverse-translational study aimed to address a unique piece of the multi-part hypothesis. (A) The 35 min zebrafish sleep analysis was designed to first identify if *Candida* infected or high fat diet treated zebrafish larvae exhibited sleep disruption when compared to their respective controls (Figures 3-6). **(B)** The post-sleep analysis qPCR method was designed to assess if *Candida* infected or high fat diet treated zebrafish larvae exhibited greater pro-inflammatory cytokine gene expression (Figures 7-8). **(C)** The sleep vs. qPCR scatterplots were designed to assess if differential sleep quality observed in either *Candida* infected or high fat diet treated zebrafish larvae was associated with pro-inflammatory cytokine gene expression (Figures 9-10). Particularly in regards to the diet-

controlled group, this experimental design was meant to assess each particular piece of the multi-part hypothesis that Western diet induced gut-dysbiosis was the trigger for pro-inflammatory cytokine mediated sleep disruption.

Our preliminary results suggest for the first time that infected zebrafish larvae *and* high fat diet treated zebrafish larvae both exhibit a greater number of sleep bouts than their respective controls as well as a possible trend (not statistically significant) for elevated IL-6 gene expression. Though future studies are necessary to compensate for the small sample size of the dietary group in the present study, these results suggest that a pro-inflammatory signaling pathway may be induced by a high fat diet in zebrafish and subsequently altering sleep disruption.

Zebrafish Sleep Analysis

In 80% of replicate sleep analysis trials, *Candida* infected larvae exhibited greater total sleep duration, sleep %, and mean sleep bout length than control larvae and, in 60% of replicate sleep analysis trials, *Candida* infected zebrafish larvae exhibited a greater number of sleep bouts than control larvae. Similarly, though no statistically significant correlations or trends were noted, high fat diet treated zebrafish larvae exhibited a greater number of sleep bouts and fragmentation index than control diet larvae. The high variability of sleep quality between control and experimentally-treated zebrafish in repeat experimental trials highlights the importance for further optimization of velocity-based sleep studies. In particular, replicating the experimental methods published by Sorribes *et*

al (2013) required several non-data producing trials due to pitfalls that were not described in previous literature[3]. Namely, the use of 1-phenyl 2-thiourea (PTU) to inhibit the pigmentation of zebrafish larvae, a common practice in zebrafish husbandry to simplify injection and imaging procedures, disrupted the ability of the Noldus Vision Device from differentiating the nearly translucent larvae from the clear background water. Thus, the protocol was modified to not include PTU treatment with zebrafish larvae up to 5 dpf. Even once pre-sleep analysis husbandry was designed in a manner such that larvae could properly be distinguished from background signal of flow-by water, high variability was observed between experimental trials. In part, this could be due to the fact that the young zebrafish larvae were not fully entrained to the 10-hour light/14-hour dark cycles and, thus, their circadian rhythm and behavioral sleep state may have been at different points during the designated sleep analysis period. However, given that previous research reports that zebrafish sleep-wake transitions decrease with age, larvae were determined to be the most appropriate model in assessing fragmentation-related variables, were more conducive to a time-restricted study, and offer a variety of physiological advantages to older zebrafish larvae (*e.g.* natural translucent appearance simplifies infection protocols). Future studies could also assess zebrafish sleep analysis at different time points during the day (*e.g.* 'morning' for early in light cycle, 'afternoon' for midway point of light cycle, etc.) to monitor light-dark entrainment or even, with more optimization, to assess 'daytime sleepiness'. While large variability may have rendered the pooled zebrafish sleep variables (Appendix C) non-statistically significant, assessing trends within biological repeat trials appeared to produce several noteworthy trends. Namely, that *Candida* infected zebrafish larvae tend to exhibit greater total sleep Duration, Sleep %,

number of sleep bouts, and mean sleep bout length when compared to non-infected larvae administered a placebo mock injection. This suggests that *Candida* infection induces sleep disruption in zebrafish larvae. Furthermore, though not observed to be statistically significant, high fat fed zebrafish larvae also exhibited a greater number of sleep Bouts as well as a larger fragmentation index. This suggests that a high fat diet is associated with a trend for sleep disruption in zebrafish larvae. The uneven control and experimental group size and lack of biological replicates likely contributed to the lack of statistically significant findings of the diet-controlled zebrafish group. In summary, despite a lack of statistical evidence for the high fat treated zebrafish group, these findings are not inconsistent with the hypothesis that a high fat diet or direct infection would induce sleep disruption in zebrafish larvae. In order to elucidate if these associative findings were mediated by pro-inflammatory cytokine signaling, the gene expression of TNF-α, IL-1β, and IL-6 were analyzed in control and treated zebrafish and then plotted against each sleep variable.

Zebrafish Inflammatory Cytokine qPCR Analysis

To investigate the our hypothesis that inflammatory signaling would disrupt sleep in zebrafish larvae, each zebrafish larvae measured for sleep analysis was subjected to single fish qPCR aimed at quantifying gene expression of the extensively studied pyrogenic and somnogenic cytokines TNF-α, IL-1β, and IL-6. The initial qPCR Trial 1 of the infected zebrafish group showed wide variability between biological replicates and errors in control gene quantification (Appendix F). This result was likely due to crude

experimental technique as subsequent repeat trials for both infected and diet-controlled were considerably more consistent and properly controlled (Appendix F, G). Using a Mann-Whitney test to compare the ΔΔCt of *Candida* infected and control zebrafish larvae, no statistically significant associations nor trends were reported. The depressed -ΔΔCt of IL-1β in Trials 1 and 2 of the infected larvae contradicted the anticipated result that IL-1β would be upregulated in the presence of fungal infection (Fradin *et al* 2007). This result, however, could be due to natural variability between zebrafish larvae and not necessarily an actual downregulation of IL-1β in infected zebrafish. What was observed in both Trials 1 and 2, as well as the pooled data set, was a non-significant increase in TNF-α and particularly IL-6 expression as gathered by the -ΔΔCt values(Figure 7). This suggests that infected zebrafish larvae are associated with both sleep disruption and a trend for elevated gene expression of inflammatory cytokines TNF-α and IL-6.

In regards to the diet-controlled zebrafish group, given the small sample size and absence of statistically significant variation between control and experimental groups, the minimal change in both TNF-α and IL-1β gene expression did not come as a surprise. However, similar to that of the infected zebrafish group, there was an increase in IL-6 -ΔΔCt as compared to control zebrafish larvae. In future studies, this experiment should attempt to be replicated to further support this preliminary observation. However, the consistent elevated gene expression of IL-6 in both *Candida* infected and high fat diet treated zebrafish larvae may support a mutual inflammatory pathway inducted by two distinct external factors.

Several statistically significant associations were noted, consistent with the hypothesis that *Candida* infection would induce sleep disruption in zebrafish. Similarly, a

non significant trend for an association correlating *Candida* infection and high fat diet treatment to elevated IL-6 gene expression was observed. However, these findings were not consistent between entire control or treated groups due to wide variability between both sleep variables and gene expression in repeat experimental trials. However, to determine if any relationship between elevated inflammatory cytokine gene expression and sleep disruption could be observed at the single fish level, each single fish ΔCt value was plotted against each single fish sleep variable (Figure 9 and 10). As indicated by more statistically relevant least squares linear regression R^2 values being observed in infected qPCR Trial 2, identifying any correlation between inflammatory cytokine gene expression and sleep variables requires tight qPCR biological replicate production. While several interesting trends were observed which suggested an association between pro-inflammatory cytokine elevated gene expression and differential sleep quality, the relatively small sample size of each single fish qPCR (ranging from 2-5 larvae) may have skewed these results. In addition to this, like humans, each zebrafish larvae is not 100% genetically identical so variation in sleep quality, response to diet, or response to infection may also be due to natural variation between fish.

FUTURE DIRECTIONS

Future work should aim both to further optimize the methods of this study as well as to attempt to elucidate more of the molecular interactions which may underlie the correlations observed between inflammatory cytokine gene expression and sleep disruption. In regards to experimental method improvements, this study should be replicated with non-velocity based zebrafish sleep analyses so that sleep variables involved in immobility, which are commonplace metrics in human sleep analysis, can be analyzed as well. That is, while metrics such as mean number of immobile phases may be assessed in humans, given that sleep was defined in zebrafish as periods of immobility, it is impossible to distinguish general sleep-like behavior from immobile phases within sleep. In addition to this, a checkpoint step between high fat feeding and inflammatory cytokine gene expression could be done, such as 16s rRNA analysis (*e.g.*Arias-Jayo, 2018) in order to confirm diet-induced gut dysbiosis prior to assessing differential inflammatory signaling. Furthermore, assessing the results of identical experimental methods on zebrafish at different stages during development, namely adult zebrafish, could prove interesting given that longer exposure to a high fat diet could have a greater impact on the state of typical gut microflora and subsequent immune response.

In regards to further research involving the molecular mechanisms underlying the relationship between diet and sleep quality in zebrafish, the most promising result of the provided study was the mutual elevation of IL-6 gene expression observed in both high fat diet treated zebrafish, who were hypothesized to be experiencing gut-dysbiosis induced inflammation, as well as *Candida* infected zebrafish, who were meant to serve as a positive control for systemic inflammation. As such, further work should first and

foremost attempt to replicate these findings in larger sample sizes and then attempt to elucidate if elevated IL-6 production is specific to any tissue(s) in infected and high fat diet treated zebrafish. Future work could even attempt to create mutant fluorescently tagged IL-6 zebrafish optimized such that, when activated, the upstream IL-6 promoter would induce gene expression of a fluorescent IL-6 protein, such that the location and fluorescence quantification of IL-6 expression in infected and diet-controlled zebrafish could be assessed via microscopy techniques. Namely, it would be interesting to observe if IL-6 expression, which would first elevate at the site of infection (*i.e.* hindbrain) or within the gut, could be observed to travel throughout the CNS in the case of hindbrain infection or travel to the CNS in the case of the gut dysbiosis mechanism.

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APPENDICES

APPENDIX A

IRB Approval Letter

Maine's Public Universities
UNIVERSITY OF MAINE SYSTEM

Jessica Aronis <jessica.aronis@maine.edu>

Continuing review 2015-11-19 - approval
3 messages

Paula Portalatin <paula.portalatin@maine.edu> Wed, Nov 20, 2019 at 9:14 AM
To: Thane Fremouw <thane.fremouw@maine.edu>
Cc: Jessica Aronis <jessica.aronis@maine.edu>

Application #: 2015-11-19
Title: Design and Development of Novel Technologies for Healthy Independent Living
PI: Thane Fremouw
Approval Period End Date: 11/25/2020

Dear Thane,

The Institutional Review Board for the Protection of Human Subjects (IRB) conducted its continuing review of the above referenced project in an expedited review on 11/20/2019. The IRB approved renewal, and the new approval period end date is noted above. The next continuing review of this project must be conducted by the IRB before the end of the approval period. Although you will receive a request for review information approximately 6-8 weeks before that date, it is your responsibility to submit review information before the approval period expires.

Attached is the approved copy of the consent document for this project. The consent forms are approved for use through **11/25/2020**. These approved copies must be duplicated and used when enrolling subjects during the approval period.

Please remember that each subject must be given a copy of the informed consent document. Any unanticipated problems or injury to the subject must be reported to the IRB. Any proposed changes to the research must be approved by the IRB prior to implementation. If you require a modification in the future, please follow these instructions.

Let me know if you have any questions, thank you.

Best regards,
Paula

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2015_11_19 Consent_forms_approved_november_2019.pdf
365K

Jessica Aronis <jessica.aronis@maine.edu> Wed, Nov 20, 2019 at 12:34 PM
To: Chris Gilbert <christopher.activas@gmail.com>
Cc: Marie Hayes <mhayes@maine.edu>

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Marie Hayes <mhayes@maine.edu> Fri, Nov 22, 2019 at 10:16 AM
To: Jessica Aronis <jessica.aronis@maine.edu>, Chris Gilbert <christopher.activas@gmail.com>

APPENDIX B

The Dietary Fat and Free Sugar - Short Questionnaire

Think about the food you've eaten over the past year. Remember breakfast, lunch, dinner and eating out. Please select the option that best describes how often you have consumed each of the following food or drink items.

		Less than 1 per month	2–3 per month	1–2 per week	3–4 per week	5+ per week
1	Mince, beef or lamb, for example, in hamburgers, nachos or bolognaise					
2	Beef or pork such as steak, ribs, roasts or in sandwiches					
3	Fried chicken or chicken burgers					
4	Sausages, frankfurts or salami					
5	Bacon					
6	Salad dressings (not low fat)					
7	Margarine, butter or oil in cooking					
8	Eggs (not egg whites alone)					
9	Pizza					
10	Cheese or cheese spread (not low fat)					
11	French fries, fried potatoes					
12	Corn chips, potato chips, popcorn with butter					
13	Doughnuts, pastries, croissants					
14	Cakes, cookies					
15	Ice cream (not sorbet or low fat)					
16	Chocolate					
17	Lollies					
18	Spreads incl. peanut butter, jam, honey					
19	Pancakes or French toast					
20	Sports drinks (e.g. Gatorade) or energy drinks (e.g. Red Bull)					
21	Soft drink (not including diet)					
22	Milk (full fat only). Include milk drunk by itself or in cappuccinos, milkshakes, hot chocolates etc.					
23	Other sweetened beverages (e.g. juice with added sugar, cordial, sweetened teas)					
24	White bread (white bread only)					
25	In the past year, how many times have you eaten food from a takeaway or fast food restaurant for example McDonalds, KFC, Mexican, Chinese, Thai, Italian (pizza or pasta)?					
		None	1–2	3–4	5–6	7+
26	In the past week, how many teaspoons of sugar have you added to your beverages, cereal or food?					

APPENDIX C

Zebrafish Sleep Variables for Infected Sleep Analysis Trials 1-6

Sleep Variables for Mock PBS Injection vs. Active *Candida* Infection Trial 1 Groups

Sleep Variable	**Mock PBS Injection (n=11)**	**Active *Candida* Infection (n=9)**	***p*-value**
Total Sleep Duration	30.72 ± 1.62	31.79 ± 3.73	**0.01831**
Number of Sleep Bouts	29.27 ± 10.67	15.22 ± 8.27	**0.002322**
Mean Sleep Bout Length	1.19 ± 0.49	2.97 ± 2.05	**0.005955**
Fragmentation Index	0.972 ± 0.419	0.519 ± 0.362	**0.009936**
Sleep %	87.86 ± 4.57	90.84 ± 10.66	**0.01831**
Total Wake Duration	4.282 ± 1.625	3.207 ± 1.358	**0.01831**
Number of Wake Bouts	30 ± 10.67	16.22 ± 7.432	**0.002322**
Mean Wake Bout Length	0.14 ± 0.03	0.164 ± 0.026	0.44038
Wake %	12.14 ± 4.57	9.159 ± 3.876	**0.01831**

Note. x ± y represents the mean ± standard deviation of the mean (SDM), (n) = sample size.

Sleep Variables for Mock PBS Injection vs. Active *Candida* Infection Trial 2 Groups

Sleep Variable	Mock PBS Injection (n=10)	Active *Candida* Infection (n=12)	*p*-value
Total Sleep Duration	13.10 ± 5.08	17.77 ± 3.61	**0.012241**
Number of Sleep Bouts	77.00 ± 10.63	82.00 ± 16.17	0.197143
Mean Sleep Bout Length	0.17 ± 0.08	0.22 ± 0.06	0.070101
Fragmentation Index	6.85 ± 2.77	4.774 ± 1.367	**0.021788**
Sleep %	37.44 ± 14.51	50.76 ± 10.31	**0.047857**
Total Wake Duration	21.90 ± 5.08	17.235 ± 2.289	**0.012241**
Number of Wake Bouts	78 ± 10.63	83 ± 16.17	0.197143
Mean Wake Bout Length	0.284 ± 0.071	0.192 ± 0.101	0.00466
Wake %	43.43 ± 14.51	52.02 ± 13.54	**0.047857**

Note. x ± y represents the mean ± standard deviation of the mean (SDM), (n) = sample size.

Sleep Variables for Mock PBS Injection vs. Active *Candida* Infection Trial 3 Groups

Sleep Variable	Mock PBS Injection (n=0)	Active *Candida* Infection (n=5)	*p*-value
Total Sleep Duration	N/A	25.58 ± 5.23	N/A
Number of Sleep Bouts	N/A	49.40 ± 16.74	N/A
Mean Sleep Bout Length	N/A	0.58 ± 0.27	N/A
Fragmentation Index	N/A	2.048 ± 0.089	N/A
Sleep %	N/A	73.08 ± 14.95	N/A
Total Wake Duration	N/A	9.42 ± 5.23	N/A
Number of Wake Bouts	N/A	50 ± 16.74	N/A
Mean Wake Bout Length	N/A	0.188 ± 0.104	N/A
Wake %	N/A	26.918 ± 14.95	N/A

Note. x ± y represents the mean ± standard deviation of the mean (SDM), (n) = sample size.

Sleep Variables for Mock PBS Injection vs. Active *Candida* Infection Trial 4 Groups

Sleep Variable	Mock PBS Injection (n=11)	Active *Candida* Infection (n=9)	*p*-value
Total Sleep Duration	19.75 ± 3.34	22.43 ± 6.24	0.116424
Number of Sleep Bouts	70.82 ± 10.68	67.33 ± 28.50	0.35553
Mean Sleep Bout Length	0.28 ± 0.06	0.58 ± 0.72	0.46812
Fragmentation Index	3.592 ± 0.747	3.498 ± 2.032	0.443771
Sleep %	56.42 ± 9.53	66.65 ± 18.94	0.111016
Total Wake Duration	15.256 ± 3.35	12.566 ± 6.242	0.116424
Number of Wake Bouts	71.818 ± 10.675	68.33 ± 28.504	0.35553
Mean Wake Bout Length	0.219 ± 0.074	0.175 ± 0.029	0.07493
Wake %	43.58 ± 9.53	35.35 ± 18.94	0.111016

Note. x ± y represents the mean ± standard deviation of the mean (SDM), (n) = sample size.

Sleep Variables for Mock PBS Injection vs. Active *Candida* Infection Trial 5 Groups

Sleep Variable	Mock PBS Injection (n=10)	Active *Candida* Infection (n=12)	*p*-value
Total Sleep Duration	26.26 ± 3.19	16.12 ± 6.84	**0.000173**
Number of Sleep Bouts	51.00 ± 17.55	76.92 ± 24.68	**0.00135**
Mean Sleep Bout Length	0.64 ± 0.44	0.51 ± 1.15	**0.00056**
Fragmentation Index	2.023 ± 0.860	5.972 ± 3.042	**0.000385**
Sleep %	75.53 ± 9.10	46.07 ± 19.53	**0.000145**
Total Wake Duration	8.738 ± 3.187	18 ± 6.843	**0.000173**
Number of Wake Bouts	52 ± 17.55	77.917 ±24.678	**0.00135**
Mean Wake Bout Length	0.168 ± 0.023	0.240 ± 0.044	**0.00027**
Wake %	24.575 ± 9.097	53.928 ± 19.525	**0.000145**

Note. x ± y represents the mean ± standard deviation of the mean (SDM), (n) = sample size.

Sleep Variables for Mock PBS Injection vs. Active *Candida* Infection Trial 6 Groups

Sleep Variable	Mock PBS Injection (n=12)	Active *Candida* Infection (n=12)	*p*-value
Total Sleep Duration	15.804 ± 6.886	15.904 ± 6.913	0.42074
Number of Sleep Bouts	74.833 ± 22.894	76.083 ± 23.907	0.38591
Mean Sleep Bout Length	0.512 ± 1.145	0.562 ± 1.322	0.48803
Fragmentation Index	5.818 ± 2.804	5.879 ± 2.867	0.479399
Sleep %	45.15 ± 19.67	45.44 ± 19.75	0.42074
Total Wake Duration	19.20 ± 6.886	19.096 ± 6.913	0.42074
Number of Wake Bouts	75.833 ± 22.894	77.083 ± 23.907	0.15386
Mean Wake Bout Length	0.249 ± 0.043	0.245 ± 0.043	0.405876
Wake %	54.845 ± 19.674	54.560 ± 19.753	0.42074

Note. x ± y represents the mean ± standard deviation of the mean (SDM), (n) = sample size.

APPENDIX D

Zebrafish Sleep Variables for High Fat Diet Sleep Analysis

Sleep Variables for Control Diet vs. High Fat Diet Trial 1 Groups

Sleep Variable	**Control Diet (n=12)**	**High Fat Diet (n=4)**	***p*-value**
Total Sleep Duration	15.989 ± 6.866	13.954 ± 3.606	0.292666
Number of Sleep Bouts	76.583 ± 23.57	84 ± 11.165	0.715805
Mean Sleep Bout Length	0.473 ± 1.014	0.172 ± 0.067	0.683516
Fragmentation Index	5.869 ± 2.858	6.446 ± 2.294	0.683516
Sleep %	45.68 ± 19.62	39.87 ± 10.3	0.952747
Total Wake Duration	19.011 ± 6.866	21.046 ± 3.606	0.292666
Number of Wake Bouts	77.583 ± 23.57	85 ± 11.165	0.715805
Mean Wake Bout Length	0.240 ± 0.045	0.247 ± 0.019	0.394092
Wake %	54.31 ±19.62	60.13 ± 10.3	0.292668

Note. x ± y represents the mean ± standard deviation of the mean (SDM), (n) = sample size.

APPENDIX E

List of Primers for qPCR

GAPDH:

Forward: 5'- TGG GCC CAT GAA AGG AAT -3'

Reverse: 5'- ACC AGC GTC AAA GAT GGA TG -3'

TNF-α:

Forward: 5'- CGC ATT TCA CAA GGC AAT TT -3'

Reverse: 5'-CTG GTC CTG GTC ATC TCT CC -3'

IL-1β:

Forward: 5'- GTC ACA CTG AGA GCC GGA AG -3'

Reverse: 5'- TGG AGA TTC CCA AAC ACA CA -3'

IL-6

Forward: 5'- CTG GTA GCC GAG AGG GGA A-3'

Reverse: 5'- CCC ATA CTG CTG AAC ACG GG -3'

APPENDIX F

Single Fish Ct, ΔCt, ΔΔCt, and Fold Change qPCR Values for Infected qPCR Trials 1 and 2

Single Fish Average Ct Values, ΔCt Values, ΔΔCt Values, and Fold Change for Mock PBS Injection vs. Active *Candida* Infection Trial 1 Group.

Gene of Interest	Single Fish ID	Single Fish Ct Values (duplicate)	Single Fish Average Ct Values	ΔCt Value	ΔΔCt Value	Fold Change
GAPDH (Control)	IA1	/, 19.13	19.14	N/A		
	IB2	21.5, 20.95	21.225	N/A		
	IC4	27.66, 21.01*		N/A		
	MA3	20.51, 17.35	18.93	N/A		
	MC2	17.14, 17.02	17.06	N/A		
	MC3	18.82, 18.9	18.86	N/A		
TNF-α	IA1	/, /				
	IB2	34.57, 31.9	33.235	12.01	-1.867	3.647
	IC4	31.37, 31.66	31.515**			
	MA3	31.06, 31.34	31.2	12.27		
	MC2	32.18, 31.4	31.79	14.73		
	MC3	32.73, 34.25	33.49	14.63		
IL-1β	IA1	26.81, /	26.81	7.37	2.203	0.217
	IB2	/, 24.25	24.25	3.025	-2.142	4.413
	IC4	23.89, 23.99	23.94**			
	MA3	23.42, 23.75	23.585	4.655		
	MC2	24.57, 25.16	24.865	7.805		
	MC3	21.75, 22.05	21.9	3.04		
IL-6	IA1	26.23, 27.22	28.725	7.585	-4.638	24.890
	IB2	24.54, 25.14	24.84	3.615	-8.608	390.046
	IC4	29.51, 28.51	58.02**			
	MA3	22.57, 36.3*				
	MC2	34.12, 33.21	33.665	16.605		
	MC3	26.22, 27.18	26.7	7.84		

Note. Single Fish IDs beginning with ‘I’ indicate Active *Candida* Infection, Single Fish IDs beginning with ‘M’ indicate Mock PBS Injection, ‘/’ indicates that no readout was provided, ‘*’ indicates that significant variation occurred between duplicate Ct Values such that Average Ct Values were not determined, ‘**’ indicates that experimental error occurred in the GAPDH Control.

Single Fish Average Ct Values, ΔCt Values, ΔΔCt Values, and Fold Change for Mock PBS Injection vs. Active *Candida* Infection Trial 2 Group.

Gene of Interest	Single Fish ID	Single Fish Ct Values (duplicate)	Single Fish Average Ct Values	ΔCt Value	ΔΔCt Value	Fold Change
GAPDH (Control)	IA1	21.87, 21.87	21.87	N/A		
	IB1	23.19, 23.74	23.46	N/A		
	MA2	21.47, 21.57	21.52	N/A		
	MB1	26.27, 26.38	21.87	N/A		
TNF-α	IA1	37.13, 38.29	37.71	15.84	-0.58	1.495
	IB1	38.89, 37.52	38.2	14.74	-1.68	3.204
	MA2	/, 37.94	37.94	16.42		
	MB1	/, /	N/A	N/A		
IL-1β	IA1	29, 28.75	28.88	7.01	2.64	0.160
	IB1	27.74, 27.47	27.6	4.14	-0.23	1.172
	MA2	27.61, 26.97	27.29	5.77		
	MB1	28.79, 29.82	29.3	2.97		
IL-6	IA1	35.28, 34.34	34.81	12.94	0.005	0.997
	IB1	33.97, 33.43	33.7	10.24	-2.695	6.476
	MA2	35.87, 35.31	35.59	14.07		
	MB1	39.09, 37.17	38.13	11.8		

Note. Single Fish IDs beginning with 'I' indicate Active *Candida* Infection, Single Fish IDs beginning with 'M' indicate Mock PBS Injection, '/' indicates that no readout was provided, '*' indicates that significant variation occurred between duplicate Ct Values such that Average Ct Values were not determined, '**' indicates that experimental error occurred in the GAPDH Control.

APPENDIX G

Single Fish Ct, ΔCt, ΔΔCt, and Fold Change qPCR Values for High Fat Diet qPCR Trials 1 and 2

Single Fish Average Ct Values, ΔCt Values, ΔΔCt Values, and Fold Change for Control Diet vs. High Fat Diet Trial 1 Group.

Gene of Interest	Single Fish ID	Single Fish Ct Values (duplicate)	Single Fish Average Ct Values	ΔCt Value	ΔΔCt Value	Fold Change
GAPDH (Control)	CB1	17.69, 17.35	17.52	N/A		
	CB2	16.59, 16.9	16.745	N/A		
	CB3	20.77, 20.63	20.7	N/A		
	CB4	23.2, 23.28	23.2	N/A		
	HB2	22.78, 22.8	22.78	N/A		
	HB3	17.18, 17.49	17.335	N/A		
	HB4	16.67, 16.68	16.675	N/A		
TNF-α	CB1	32.21, 30.37	31.29	13.77		
	CB2	30.82, 30.96	30.89	14.145		
	CB3	33.88, 34.14	34.01	13.31		
	CB4	35.08, 35.51	35.295	12.095		
	HB2	34.5, 34.75	34.625	11.845	-1.485	2.799
	HB3	31.75, 31.65	31.7	14.365	1.035	0.488
	HB4	31.39, 30.22	30.805	14.13	0.8	0.574
IL-1β	CB1	23.17, 22.78	22.975	5.455		
	CB2	22.02, 21.81	21.915	5.17		
	CB3	22.97, 22.63	22.8	2.1		
	CB4	24.04, 24.09	24.065	0.865		
	HB2	22.9, 22.85	22.875	0.095	-3.303	9.866
	HB3	24, 24.28	24.14	6.805	3.408	0.094
	HB4	23.68, 23.4	23.54	6.865	3.468	0.090
IL-6	CB1	34.29, 31.63	32.96	15.44		
	CB2	29.51, 26.6	28.055	11.31		
	CB3	26.79, 26.6	26.695	5.995		
	CB4	27.97, 28.29	28.13	4.93		

Table continued

HB2	27.23, 27.78	27.505	4.725	-4.693	25.880
HB3	24.11, 25.84	24.975	7.64	-1.779	3.431
HB4	25.99, 23.97	24.98	8.305	-1.114	2.164

Note. Single Fish IDs beginning with 'C' indicate Control Diet, Single Fish IDs beginning with 'H' indicate High Fat Diet, '/' indicates that no readout was provided, '*' indicates that significant variation occurred between duplicate Ct Values such that Average Ct Values were not determined, '**' indicates that experimental error occurred in the GAPDH Control.

Single Fish Average Ct Values, ΔCt Values, ΔΔCt Values, and Fold Change for Control Diet vs. High Fat Diet Trial 2 Group.

Gene of Interest	Single Fish ID	Single Fish Ct Values (duplicate)	Single Fish Average Ct Values	ΔCt Value	ΔΔCt Value	Fold Change
GAPDH (Control)	CB1	22.93, 21.14	22.035	N/A		
	CB2	19.75, 20.09	19.92	N/A		
	CB3	20.01, 19.4	19.705	N/A		
	CB4	19.3, 18.78	19.3	N/A		
	HB4	17.65, 17.3	17.65	N/A		
	HC1	17.44, 17.4	17.42	N/A		
	HC2	16.85,17.04	16.945	N/A		
TNF-α	CB1	33.03, 33.25	33.14	11.105		
	CB2	33.06, 32.91	32.985	13.605		
	CB3	34.72, 37.36	36.04	16.335		
	CB4	32.37, 32.1	32.235	12.945		
	HB4	30.81, 30.73	30.77	13.12	-0.24	1.181
	HC1	30.99, 31.96	31.475	14.055	0.695	0.618
	HC2	30.27, 30.76	30.515	13.57	0.21	0.865
IL-1β	CB1	27.58, 30.23	28.905	6.87		
	CB2	32.08, 27.56	29.82	9.9		
	CB3	30.17, 28.05	29.11	9.405		
	CB4	27.05, 26.29	26.67	7.37		
	HB4	24.04, 24.01	24.025	6.375	-2.011	4.031
	HC1	24.35, 24.47	24.41	6.99	-1.400	2.632
	HC2	23.56, 23.35	23.455	6.51	-1.876	3.671
IL-6	CB1	32.72, 34.1	33.41	11.375		
	CB2	31.33, 29.55	30.44	10.52		
	CB3	30.84, 27.75	29.295	9.59		
	CB4	24.86, 24.33	24.595	5.295		
	HB4	24.03, 23.2	23.615	5.965	-3.23	9.383
	HC1	23.11, 24.2	23.655	6.235	-2.96	7.781
	HC2	24.11, 22.6	23.55	6.41	-2.785	6.892

Note. Single Fish IDs beginning with 'C' indicate Control Diet, Single Fish IDs beginning with 'H' indicate High Fat Diet, '/' indicates that no readout was provided, '*' indicates that significant variation occurred between duplicate Ct Values such that Average Ct Values were not determined, '**' indicates that experimental error occurred in the GAPDH Control.

APPENDIX H

Whole-study Zebrafish Sleep Variable Summary

Single fish sleep variables for infected analysis group (Trials 1-6) and diet analysis group (Trial 1).

	Fish ID	Total sleep duration	# Sleep bouts	Mean sleep bout length	Fragmentation index	Sleep %	Total wake duration	# wake bouts	Mean wake bout length	Wake %
Control	*A1*	32.920	15.000	131.67	0.456	94.050	2.080	16.000	0.130	5.950
Trial 1	*A2*	32.380	22.000	88.320	0.679	92.520	2.620	23.000	0.114	7.480
	A3	31.320	27.000	69.590	0.862	89.480	3.680	28.000	0.131	10.520
	A4	29.420	35.000	50.430	1.190	85.050	5.580	36.000	0.155	14.950
	B1	26.900	53.000	30.450	1.970	76.860	8.100	54.000	0.150	23.140
	B2	/	/	/	/	/	/	/	/	/
	B3	30.950	32.000	58.030	1.034	88.430	4.050	33.000	0.123	11.570
	B4	30.980	29.000	64.100	0.936	88.520	4.020	30.000	0.134	11.480
	C1	31.200	17.000	110.12	0.545	89.140	3.800	18.000	0.211	10.860
	C2	31.630	24.000	75.080	0.759	90.380	3.370	25.000	0.135	9.620

	C3	29.780	39.000	45.820	1.309	85.100	5.220	40.000	0.131	14.900
	C4	30.420	29.000	62.930	0.953	86.900	4.580	30.000	0.153	13.100
Infected	*A1*	22.350	29.000	46.240	1.298	63.860	12.650	30.000	0.422	36.140
Trial 1	*A2*	33.180	12.000	165.92	0.398	94.810	1.820	13.000	0.140	5.190
	A3	/	/	/	/	/	/	/	/	/
	A4	/	/	/	/	/	/	/	/	/
	B1	34.300	5.000	411.60	0.146	98.000	0.700	6.000	0.117	2.000
	B2	33.730	11.000	184.00	0.326	96.380	1.270	12.000	0.106	3.620
	B3	34.280	6.000	342.83	0.175	97.950	0.720	7.000	0.103	2.050
	B4	31.430	21.000	89.810	0.668	89.810	3.570	22.000	0.162	10.190
	C1	30.980	25.000	74.360	0.807	88.520	4.020	26.000	0.155	11.480
	C2	/	/	/	/	/	/	/	/	/
	C3	33.520	12.000	167.58	0.358	95.760	1.480	13.000	0.114	4.240
	C4	32.370	16.000	121.38	0.494	92.480	2.630	17.000	0.155	7.520
Control	*A1*	8.270	76.000	6.530	9.190	23.620	26.730	77.000	0.347	76.380
Trial 2	*A2*	12.420	79.000	9.430	6.480	35.690	22.580	80.000	0.282	64.310
	A3	11.080	92.000	7.230	8.300	31.65	23.920	93.00	0.257	68.350

					0		0		
	A4	7.570	79.000	5.750	10.440	21.620	27.430	80.000	0.343	78.380
	B1	13.750	93.000	8.870	6.760	39.270	21.250	94.000	0.226	60.730
	B2	20.630	59.000	20.980	2.860	58.930	14.370	60.000	0.240	41.070
	B3	5.830	66.000	5.300	11.320	16.650	29.170	67.000	0.435	83.350
	B4	12.250	81.000	9.070	6.610	35.000	22.750	82.000	0.277	65.000
	C1	18.270	85.000	12.900	4.650	52.200	16.730	86.000	0.195	47.800
	C2	18.270	62.000	17.680	3.390	52.200	16.730	63.000	0.266	47.800
	C3	9.070	75.000	7.260	8.270	25.910	25.930	76.000	0.341	74.090
	C4	19.800	77.000	15.430	3.890	56.570	15.200	78.000	0.195	43.430
infected	*A1*	13.150	43.000	18.350	3.270	37.570	21.850	44.000	0.497	62.430
Trial 2	*A2*	11.670	85.000	8.240	7.280	33.340	23.330	86.000	0.271	66.660
	A3	/	/	/	/	/	/	/	/	/
	A4	/	/	/	/	/	/	/	/	/
	B1	15.970	99.000	9.680	6.200	45.630	19.030	100.000	0.190	54.370
	B2	21.550	75.000	17.240	3.480	61.570	13.450	76.000	0.177	38.430
	B3	20.970	84.000	14.980	4.010	59.910	14.030	85.000	0.165	40.090

	B4	16.280	91.000	10.730	5.590	46.510	18.720	92.000	0.203	53.490
	C1	19.670	78.000	15.130	3.970	56.200	15.330	79.000	0.194	43.800
	C2	22.770	77.000	17.740	3.380	65.060	12.230	78.000	0.157	34.940
	C3	17.870	100.00	10.720	5.600	51.060	17.130	101.000	0.170	48.940
	C4	17.750	88.000	12.100	4.960	23.000	17.250	89.000	0.194	77.000
Control	*A1*	/	/	/	/	/	/	/	/	/
Trial 3	*A2*	/	/	/	/	/	/	/	/	/
	A3	/	/	/	/	/	/	/	/	/
	A4	/	/	/	/	/	/	/	/	/
	B1	/	/	/	/	/	/	/	/	/
	B2	/	/	/	/	/	/	/	/	/
	B3	/	/	/	/	/	/	/	/	/
	B4	/	/	/	/	/	/	/	/	/
	C1	/	/	/	/	/	/	/	/	/
	C2	/	/	/	/	/	/	/	/	/
	C3	/	/	/	/	/	/	/	/	/
	C4	/	/	/	/	/	/	/	/	/
Infected	*A1*	/	/	/	/	/	/	/	/	/
Trial 3	*A2*	/	/	/	/	/	/	/	/	/
	A3	/	/	/	/	/	/	/	/	/

	A4	/	/	/	/	/	/	/	/
	B1	22.800	73	18.740	3.200	65.170	12.200	74.000	0.165	34.830
	B2	25.970	60	25.970	2.310	74.190	9.030	61.000	0.148	25.810
	B3	18.220	44	24.800	2.430	52.050	16.780	45.000	0.373	47.950
	B4	/	/	/	/	/	/	/	/	/
	C1	/	/	/	/	/	/	/	/	/
	C2	30.620	33	55.670	1.080	87.480	4.380	34.000	0.129	12.520
	C3	/	/	/	/	/	/	/	/	/
	C4	30.280	37	49.100	1.220	86.520	4.720	38.000	0.124	13.480
Control	*A1*	21.130	74.000	17.140	3.502	60.380	13.870	75.000	0.185	39.620
Trial 4	*A2*	19.280	83.000	13.940	3.304	55.100	15.720	84.000	0.187	44.900
	A3	18.530	83.000	13.400	4.478	52.950	16.470	84.000	0.196	47.050
	A4	11.420	57.000	12.020	4.991	32.620	23.580	58.000	0.407	67.380
	B1	18.950	83.000	13.700	4.380	54.140	16.050	84.000	0.191	45.860
	B2	23.170	68.000	20.440	2.935	66.190	11.830	69.000	0.171	33.810
	B3	18.270	53.000	20.680	2.901	52.190	16.730	54.000	0.310	47.810
	B4	20.560	79.000	15.620	3.841	58.760	14.440	80.000	0.181	41.240

	C1	20.730	71.000	17.520	3.492	59.240	14.270	72.000	0.198	40.760
	C2	20.830	62.000	20.160	2.976	59.520	14.170	63.000	0.225	40.480
	C3	/	/	/	/	/	/	/	/	/
	C4	24.320	66.000	22.110	2.714	69.480	10.680	67.000	0.159	30.520
infected	*A1*	33.050	14.000	141.60	0.424	99.430	1.950	15.000	0.130	0.570
Trial 4	*A2*	17.150	90.000	11.430	5.248	49.000	17.850	91.000	0.196	51.000
	A3	/	/	/	/	/	/	/	/	/
	A4	/	/	/	/	/	/	/	/	/
	B1	24.020	75.000	19.210	3.123	68.620	10.980	76.000	0.144	31.380
	B2	22.200	68.000	19.590	3.063	63.430	12.800	69.000	0.186	36.570
	B3	/	/	/	/	/	/	/	/	/
	B4	30.520	29.000	63.140	0.950	87.190	4.480	30.000	0.149	12.810
	C1	19.350	87.000	13.340	4.496	55.290	15.650	88.000	0.178	44.710
	C2	14.420	93.000	9.300	6.451	41.190	20.580	94.000	0.219	58.810
	C3	17.230	90.000	11.490	5.222	49.240	17.770	91.000	0.195	50.760
	C4	23.970	60.000	23.970	2.503	68.480	11.030	61.000	0.181	31.520
Control	*A1*	25.970	59.000	26.410	2.272	74.190	9.030	60.000	0.151	25.810

Trial 5	*A2*	30.730	25.000	73.760	0.813	87.810	4.270	26.000	0.164	12.190
	A3	26.780	46.000	34.930	1.717	76.520	8.220	47.000	0.175	23.480
	A4	27.670	45.000	36.890	1.627	79.050	7.330	46.000	0.159	20.950
	B1	26.020	58.000	26.910	2.229	74.330	8.980	59.000	0.152	25.670
	B2	24.030	67.000	21.520	2.788	68.670	10.970	68.000	0.161	31.330
	B3	31.600	19.000	99.790	0.526	90.290	3.400	20.000	0.170	9.710
	B4	/	/	/	/	/	/	/	/	/
	C1	21.230	59.000	21.590	2.779	60.670	13.770	60.000	0.230	39.330
	C2	/	/	/	/	/	/	/	/	/
	C3	23.170	74.000	18.780	3.194	66.190	11.830	75.000	0.158	33.810
	C4	25.420	58.000	26.290	2.282	77.620	9.580	59.000	0.162	22.380
infected	*A1*	19.170	72.000	15.970	3.756	54.760	15.830	73.000	0.217	45.240
Trial 5	*A2*	14.500	70.000	12.430	4.828	41.430	20.500	71.000	0.289	58.570
	A3	10.670	106.000	6.030	9.938	30.480	24.330	107.000	0.227	69.520
	A4	11.850	87.000	8.170	8.817	33.860	23.150	88.000	0.263	66.140
	B1	16.520	94.000	10.540	5.691	47.190	18.480	95.000	0.195	52.810
	B2	16.830	89.000	11.350	5.287	48.10	18.170	90.00	0.202	51.900

					0		0		
	B3	10.380	87.000	7.160	8.379	29.670	24.620	88.000	0.280	70.330
	B4	20.700	68.000	18.270	3.285	59.140	14.300	69.000	0.207	40.860
	C1	20.650	70.000	17.700	3.390	59.000	14.350	71.000	0.202	41.000
	C2	8.670	79.000	6.580	9.115	24.760	26.330	80.000	0.329	75.240
	C3	33.170	8.000	248.7	0.241	94.760	1.830	9.000	0.203	5.240
	C4	10.300	93.000	6.710	8.942	29.710	24.700	94.000	0.263	70.290
Control	*A1*	19.100	71.000	0.269	3.717	54.571	15.900	72.000	0.221	45.429
Trial 6	*A2*	14.283	79.000	0.181	5.531	40.810	20.717	80.000	0.259	59.190
	A3	10.717	89.000	0.120	8.305	30.619	24.283	90.000	0.270	69.381
	A4	11.783	86.000	0.137	7.298	33.667	23.217	87.000	0.267	66.333
	B1	15.983	93.000	0.172	5.819	45.667	19.017	94.000	0.202	54.333
	B2	16.683	90.000	0.185	5.395	47.667	18.317	91.000	0.201	52.333
	B3	9.700	86.000	0.113	8.866	27.714	25.300	87.000	0.291	72.286
	B4	20.183	65.000	0.311	3.220	57.667	14.817	66.000	0.225	42.333
	C1	19.950	68.000	0.293	3.409	57.000	15.050	69.000	0.218	43.000

	C2	8.417	78.000	0.108	9.267	24.048	26.583	79.000	0.336	75.952
	C3	33.133	8.000	4.142	0.241	94.667	1.867	9.000	0.207	5.333
	C4	9.717	85.000	0.114	8.748	27.762	25.283	86.000	0.294	72.238
infected	*A1*	18.650	71.000	0.263	3.807	53.286	16.350	72.000	0.227	46.714
Trial 6	*A2*	14.150	79.000	0.179	5.583	40.429	20.850	80.000	0.261	59.571
	A3	10.350	97.000	0.107	9.372	29.571	24.650	98.000	0.252	70.429
	A4	11.617	84.000	0.138	7.231	33.190	23.383	85.000	0.275	66.810
	B1	16.300	94.000	0.173	5.767	46.571	18.700	95.000	0.197	53.429
	B2	16.717	89.000	0.188	5.324	47.762	18.283	90.000	0.203	52.238
	B3	10.017	87.000	0.115	8.686	28.619	24.983	88.000	0.284	71.381
	B4	20.400	67.000	0.304	3.284	58.286	14.600	68.000	0.215	41.714
	C1	20.600	70.000	0.294	3.398	58.857	14.400	71.000	0.203	41.143
	C2	8.650	76.000	0.114	8.786	24.714	26.350	77.000	0.342	75.286
	C3	33.283	7.000	4.755	0.210	95.095	1.717	8.000	0.215	4.905
	C4	10.117	92.000	0.110	9.094	28.905	24.883	93.000	0.268	71.095
Control	*A1*	18.983	74.000	0.257	3.898	54.240	16.017	75.000	0.214	45.760

Trial 1	*A2*	14.383	78.000	0.184	5.423	41.100	20.617	79.000	0.261	58.900
	A3	10.683	99.000	0.108	9.267	30.520	24.317	100.000	0.243	69.480
	A4	11.767	86.000	0.137	7.309	33.620	23.233	87.000	0.267	66.380
	B1	16.400	94.000	0.174	5.732	46.860	18.600	95.000	0.196	53.140
	B2	16.800	89.000	0.189	5.298	48.000	18.200	90.000	0.202	52.000
	B3	10.000	87.000	0.115	8.700	28.570	25.000	88.000	0.284	71.430
	B4	20.383	66.000	0.309	3.238	58.240	14.617	67.000	0.218	41.760
	C1	20.570	69.000	0.298	3.355	58.762	14.430	70.000	0.206	41.238
	C2	8.583	77.000	0.111	8.971	24.520	26.417	78.000	0.339	75.480
	C3	33.167	9.000	3.685	0.271	94.760	1.833	10.000	0.183	5.240
	C4	10.150	91.000	0.112	8.966	29.000	24.850	92.000	0.270	71.000
High Fat	*A1*	18.850	72.000	0.262	3.820	53.857	16.150	73.000	0.221	46.143
Trial 1	*A2*	14.400	79.000	0.182	5.486	41.143	20.600	80.000	0.258	58.857
	A3	10.750	98.000	0.110	9.116	30.714	24.250	99.000	0.245	69.286
	A4	11.817	87.000	0.136	7.362	33.762	23.183	88.000	0.263	66.238

AUTHOR'S BIOGRAPHY

Ben Williams was born in Portland, ME and raised in Cumberland Center, ME where he graduated from Greely High School in 2017. Ben is a Biochemistry & Microbiology double major. While at the University of Maine, Ben has served as the President of The Senior Skull Honor Society, the Vice President of Operation H.E.A.R.T.S., and the Event Coordinator for Best Buddies UMaine Chapter. In addition to this, Ben has worked for the University Volunteer Ambulance Corps for two years before working as an EMT for Northern Light Medical Transport and Emergency Care in both Bangor and Ellsworth. Ben spent two years within the Molloy Lab and then a year within both the Hayes Lab and the Wheeler Lab where he collected the data presented within this thesis. While Ben was accepted to the Tufts University School of Medicine - Maine Medical Center Maine Track Program via an Early Assurance Program, he has decided to delay his medical school education to pursue other professional and personal interests. After graduation, he will be continuing research as a Research Technician at Boston University Medical Center within the Logan Lab in the Department of Pharmacology and Experimental Therapeutics.